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Graeme I. Murray *Editor*

Laser Capture Microdissection

Methods and Protocols

Third Edition

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Edited by

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Preface

Laser microdissection techniques have revolutionized the ability of researchers in general and pathologists in particular to carry out molecular analysis on specific types of normal and diseased cells and to fully utilize the power of current molecular technologies including PCR, microarrays, next generation sequencing, and proteomics. The primary purpose of the third edition of this volume of *Methods in Molecular Biology* is to provide the reader with practical advice on how to carry out tissue-based laser microdissection successfully in their own laboratory using the different laser microdissection systems that are available and apply a wide range of molecular technologies. The individual chapters encompass detailed descriptions of the individual laser-based microdissection systems. The downstream applications of the laser microdissected tissue described in the book include next generation sequencing as well as gene expression analysis including application to microarrays and proteomics. The editor is especially grateful to all the contributing authors for the considerable time and effort they have put into the individual chapters.

The series editor, John Walker, has provided expert guidance through the editorial process while colleagues at Springer have been very helpful in dealing with all the publication-related issues.

Aberdeen, Scotland, UK *Graeme I. Murray*

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Chapter 1

Laser Capture Microdissection: Insights into Methods and Applications

Meera Mahalingam

Abstract

Laser capture microdissection is a non-molecular, minimally disruptive method to obtain cytologically and/or phenotypically defined cells or groups of cells from heterogeneous tissues. Its advantages include efficient rapid and precise procurement of cells. The potential disadvantages include time consuming, expensive, and limited by the need for a pathologist for recognition of distinct subpopulations within a specified sample. Overall it is versatile allowing the preparation of homogenous isolates of specific subpopulations of cells from which DNA/RNA or protein can be extracted for RT-PCR, quantitative PCR, next-generation sequencing, immunoblot blot analyses, and mass spectrometry.

Key words Gene expression, Immunoblot, Laser microdissection, Next-generation sequencing, PCR, Proteomics

1 Introduction

The molecular analysis of DNA, RNA, and protein derived from diagnostic tissue has revolutionized pathology and led to the identification of a broad range of diagnostic and prognostic markers [1]. Analysis of critical gene expression and protein patterns in normal developing and diseased tissue progression requires the microdissection and extraction of a microscopic homogeneous cellular subpopulation from its complex tissue milieu [2]. However, the reliability of tests based on tissue or cell extracts often depends crucially on the relative abundance of the cell population in question [1]. Therefore, a prerequisite for modern molecular research is the capability of preparing pure samples without a large number of "contaminating" cells [1, 3]. Laser capture microdissection (LCM) offers a simple, one-step process that provides scientists with a fast and dependable method of preserving and isolating single cells, or clusters of cells, from tissue sections by direct microscopic visualization [2, 4, 5].

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2 History

The need to isolate specific cells from complex tissues in order to carry out accurate molecular assays has been argued for decades [6]. In the 1970s, Lowry and Passonneau pioneered a procedure for biochemical microanalysis, which utilized "freehand" microdissection of specific cell types under a microscope [6, 7]. At the same time, several papers described different techniques that were also based on manual dissection (under microscope control) using razor blades, needles, or fine glass pipettes to isolate the cells of interest [6]. An obvious shortcoming is that manual microdissection is time-consuming, tedious, and does not allow for precise control of the material effectively selected [6, 7].

A significant technological advance was proposed by Shibata in 1993 who suggested selective ultraviolet radiation fractionation (SURF), a procedure that utilized an ultraviolet laser beam to destroy the DNA of all undesired components of the tissue, while the cells of interest were protected by a specific dye [6–8]. Unfortunately, this technique is only useful for analytes that are susceptible to degradation by UV-light, such as DNA [7]. Subsequent improvements of this procedure led to the development of more sophisticated techniques that enabled isolation of single cells [6].

The laser capture microdissection system (LCM) was developed during the mid-1990s by Dr. Emmert-Buck and colleagues at the National Institutes of Health (NIH), Bethesda, Maryland, USA [9]. The system was initially developed for the analyses of solid tumors, and was later commercialized by Arcturus Engineering (Sunnyvale, CA, USA) as the PixCell system [6, 9]. The Pixcell series is currently the most widely used laser-based microdissection system, its development propelled by its integration into the "cancer genome anatomy project" (CGAP) sponsored by the National Cancer Institute (NCI) [1, 9]. Multiple generations of this instrument (Pixcell II; Arcturus Engineering, Mountain View, CA, USA) are currently on the market [1]. Arcturus has also recently commercialized a new system (Veritas™ microdissection) that combines their LCM system, based on infrared laser, with UV laser cutting possibilities, the latter ideal for non-soft tissues and capturing large numbers of cells [6, 10].

3 Principle

The LCM system by Arcturus (PixCell II) is based on the selective adherence of visually targeted cells and tissue fragments to a special thermoplastic film made of an ethylene vinyl acetate membrane (EVA) activated by a low energy infrared laser pulse [1, 6]. The system consists of an inverted microscope, a solid state near infrared

laser diode, a laser control unit, a joystick controlled microscope stage with a vacuum chuck for slide immobilization, a CCD (charge coupled device) camera, and a color monitor. The LCM microscope is usually connected to a personal computer for additional laser control and image archiving [1]. The thermoplastic membrane used for transfer of selected cells is manufactured on the bottom surface of a plastic support cap, which acts as an optic for focusing the laser [1, 11]. It has a diameter of approximately 6 mm and fits on standard 0.5 ml microcentrifuge tubes to facilitate further tissue processing [1].

The cap is suspended on a mechanical transport arm and placed on the desired area of the mounted tissue sections [1]. After visual selection of the desired cells, laser activation leads to focal melting of the EVA membrane, which has its absorption maximum near the wavelength of the laser [1]. The polymer melts only in the vicinity of the laser, and expands into the section filling small hollow spaces present in the tissue [1, 11]. Properly melted polymer spots have a dark outer ring and a clear center, indicating that the polymer has melted and is in direct contact with the slide (Fig. 1) [11]. The polymer then re-solidifies within milliseconds (ms) and forms a composite with the tissue [1]. A dye incorporated into the polymer serves two purposes: first, it absorbs laser energy, preventing damage to the cellular constituents, and second, it aids in visualizing areas of melted polymer [11]. The adherence of the tissue to the activated membrane exceeds the adhesion to the glass slide and allows for selective removal of the desired cells [1]. Laser pulses between 0.5 and 5 ms in duration repeated multiple times across the cap surface, allow for rapid isolation of large numbers of cells [1]. Lifting the cap then shears the selected cells from the heterogeneous tissue section [1, 11].

The minimum diameter of the laser beam (7.5 μm) has been reduced in the newer generation machine. Under standard working conditions, the area of the polymer melting corresponds exactly to

Fig. 1 LCM polymer bubbles: Properly melted polymer bubbles have a dark outer ring, indicating the polymer has melted and is in direct contact with the slide. (**a**) Larger spots can be created by increasing the power and spot size of the laser to 100 mW and 30 μm respectively. (**b**) Smaller spots can be created by decreasing the power and spot size of the laser to 30 mW and 10 μm respectively

the laser spot size. Also, since most of the energy is absorbed by the membrane, the maximum temperatures reached by the tissue upon laser activation are in the range of 90 °C for several milliseconds, thus leaving biological macromolecules intact [1]. The short laser pulse durations used (0.5–5.0 ms), the low laser power levels required (1–100 mW), the absorption of the laser pulse by the dye-impregnated polymer, and the long elapsed time (0.2 μs) between laser pulses combine to prevent any significant amount of heat deposition at the tissue surface which might compromise the quality of the tissue/cells utilized in later laboratory analyses [1, 9, 11].

4 Tissue Fixation, Sectioning, and Staining

Laser-based microdissection techniques have been applied to a wide range of tissues, prepared with a variety of methods, and utilizing a diverse range of biological samples [9]. However, the procedures used in the preparation of tissue or cells for microdissection vary with the intended purposes and the analytes sought [7]. Tissue specimens are typically either fixed in aldehyde-based fixatives (e.g., 10% formalin) or snap frozen [12].

Formalin-fixation (10% buffered formaldehyde) is the standard for morphologic preservation of tissue, and has been used in histology laboratories for decades because of its low cost and rapid, complete penetration of tissue [7, 11]. Although formalin-fixed tissues are well preserved for histopathological evaluation, the quality of the macromolecules is severely compromised [12]. It is an "additive" fixative that creates cross-links between itself and proteins, and between nucleic acids and proteins [6]. This cross-linking interferes with recovery of nucleic acids and proteins, as well as the amplification of DNA and RNA by polymerase chain reaction (PCR) [6, 7]. As a consequence of these cross-links, the nucleic acids isolated from these specimens are highly fragmented, especially as fixation time is increased [6]. This problem often occurs when using archival material, especially since pathology laboratories did not pay much attention to fixation times in the past [6]. Fortunately, it has been shown that shorter lengths of DNA, up to approximately 200 base pairs, are recoverable by PCR after the extraction from formalin-fixed paraffin-embedded (FFPE) tissue [7].

Ethanol-based fixatives offer the best RNA preservation by fixing tissues through dehydration without creating chemical links [6, 7]. However, it has been found that sectioning with alcohol-based fixatives is more difficult [13]. Therefore, the use of alcohol fixatives is only feasible if microdissection is considered one of the possible options for processing the sample from the start [6].

In the case of histological preparations, it is certainly better to utilize samples that have been snap-frozen and stored in liquid nitrogen at 80 °C or colder [6, 7]. Frozen sections do not undergo cross-linking due to fixatives, and as a result, yield high-quality messenger RNA (mRNA) and proteins [6, 12]. However, freezing and cryostat sectioning can significantly disrupt the histological architecture of the tissue [12]. This is a major problem since LCM is accomplished through identification of cells by morphological characteristics [11].

The main goal of tissue preparation is to ensure that both the morphology of the tissue and molecules of interest are preserved [9]. Recently, methods have been developed for the extraction and amplification of RNA from FFPE tissue sections [14]. Like fresh tissue, mRNA amplification by nested RT-PCR (reverse-transcriptase PCR) has been reported for single cells isolated from FFPE tissue through LCM [1, 15]. Similarly, there has been a development of protocols which permit the extraction and mass spectrometric analysis of proteins from FFPE tissues [9]. However, even though new technologies are being developed to reverse cross-linking for extraction of sufficient quantities of nucleic acids and proteins, high-quality yield of RNA and proteins is best achieved with frozen or ethanol-fixed tissue [11]. The ability to effectively break the cross-links in nucleic acid caused by formalin could allow the utilization of a wealth of archived FFPE tissue for RNA expression and genomic analysis [7].

Optimal LCM is achieved with tissue sections cut at a thickness of 2–15 μm [11]. Tissue sections thinner than 5 μm may not provide full cell thickness, necessitating multiple microdissections in order to obtain an adequate number of cells for a given assay [11]. Tissue sections thicker than 15 μm may not microdissect completely, leaving integral cellular components adhering to the slide [11].

Ideally, staining should provide an acceptable morphology to allow the selection of target cells without interfering with the macromolecules of interest, or subsequent molecular techniques [6]. Therefore, tissue sections should be exposed to the dye solution for the briefest period of time [9, 11]. Minimal staining times limit potential protein alterations, and reduce the risk of chemical modification due to contact with reagents [9, 11]. Sections can be stained satisfactorily by a few seconds exposure to the dye solution, followed by removal of excess dye with rapid washing [9]. Examples of LCM-compatible stains are Hematoxylin and Eosin (most commonly used for examination of histologic sections), methylene blue, Wright-Giemsa, and toluidine blue [7, 11]. In our experience, eosin staining is not necessary for visualization of cells. Specimens can also be stained immunohistochemically or with fluorescent labels, allowing the investigator to target cells based on the presence of specific antigens [7, 9]. Stained sections are dehydrated and kept without a coverslip [6].

4.1 Factors Affecting Yield of DNA, RNA, and Protein

These include quality of sample, time of preservation before microdissection, type of preservation, fixation method, and efficiency of microdissection [2]. In our experience, fixation is *the* most critical step to ensure a high-quality yield of DNA, RNA, or protein [11]. Quality of fixation is dependent on the length of time for fixative penetration in the tissue, temperature of fixation, and tissue size [2]. In contrast to DNA, mRNA and protein are more sensitive to fixation, are quickly degraded, and require stringent RNase and proteinase free conditions during specimen handling and preparation [1, 2]. Therefore, the longer the fixative takes to penetrate the tissue, the greater the chance of RNA or protein degradation due to these ubiquitous RNases and proteinases [2]. As a result, tissue microdissection is currently more widely employed in the analysis of DNA, as opposed to RNA and proteins, which are much more sensitive to degradation and fixation [6].

In general, one set of microdissected cells is used for the downstream analysis of only one type of molecule [2]. Each class of molecules requires different solubilization schemes, extraction buffers, and denaturing temperatures. For example, a population of 10,000 microdissected cells could be solubilized in denaturing buffer at 70 °C for downstream protein analysis, while a second set of 100 cells could be treated with proteinase K at 65 °C for downstream DNA analysis [2]. Captured cells are detached from the cap membrane by proteinase digestion, and standard single-step PCR protocols can be applied if enough cells have been collected [1]. As can be seen, it is often necessary to microdissect many more cells than necessary based solely on DNA, RNA, or protein content of a cell [11]. Examples of cellular yield required for DNA, RNA, and protein analyses are greatly varied, and range from 100 to 2000 cells for DNA, 5000–10,000 cells for RNA, and up to 4000–200,000 cells for protein analyses [2].

4.2 Evolution of LCM Protocols

Due to the infancy of LCM technology, protocols have been constantly changing. Our own experience confirms this. At the outset, tissue slides were cut in 7–10 μm sections and mounted on uncharged slides. However, we found that 5 μm sections allowed for better procurement of cells, particularly in melanoma samples (Fig. 2). It is our contention that in this entity, thinner tissue sections allowed the melted polymer to more effectively penetrate tissue samples, thus enhancing yield. Similarly, modifications in LCM power and spot size have led to more efficient tissue retrieval. Initially, the Pixcell IIe LCM machine was used with a power ranging from 70 to 100 mW and a spot size of 10 μm. However, after numerous trials, we found that a power of 80 mW and a spot size of 7.5 μm were most effective in optimizing our yield.

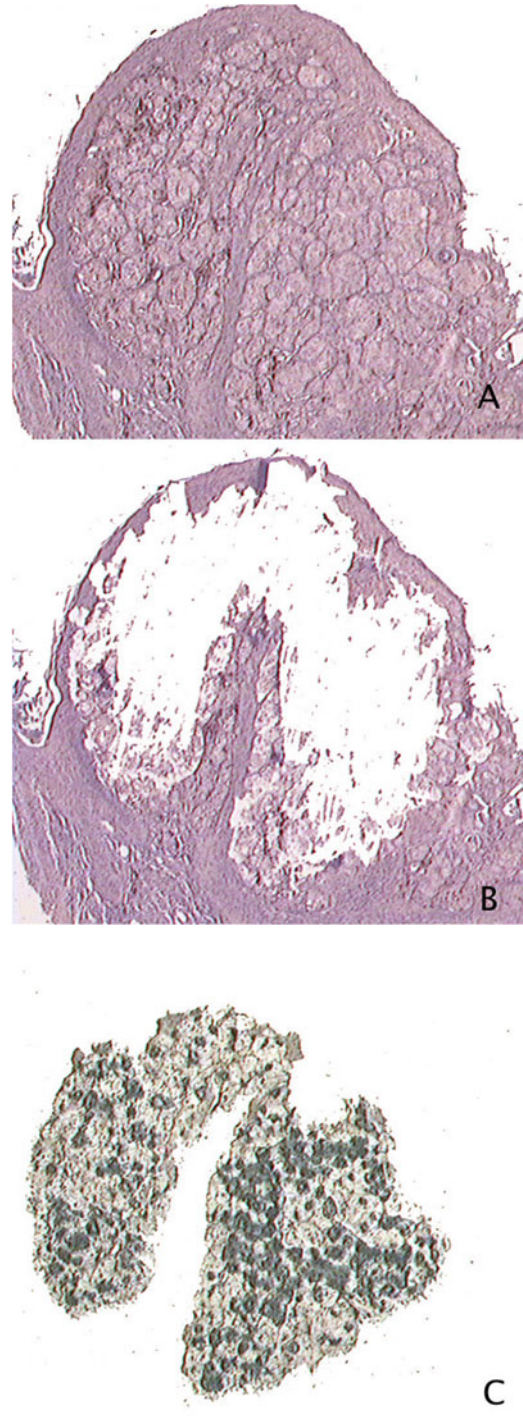

Fig. 2 LCM of melanoma: (**a**) Melanoma nested in heterogeneous tissue section prior to LCM (40× magnification). (**b**) Melanoma after LCM. (**c**) Melted polymer bubble containing melanoma cells extracted from the heterogeneous tissue section

5 Utility

5.1 Advantages

Perhaps the most relevant advantage of LCM is its speed while maintaining precision and versatility [1]. LCM provides a reliable method to procure pure, precise populations of target cells from a wide range of cell and tissue preparations via microscopic visualization [16]. The LCM system is applicable to normal glass slides (along with a wide range of other preparations), allowing routinely prepared material to be used after the removal of the coverslip [6]. Conventional techniques for molecular analysis are based on whole tissue dissociation and therefore introduce inherent contamination problems, thus reducing the specificity and sensitivity to subsequent molecular analysis, while requiring a high level of manual dexterity. LCM, on the other hand, is a "no touch" technique that does not destroy adjacent tissues following initial microdissection. This allows several tissue components to be sampled sequentially from the same slide (for example, normal and atypical cells) [1, 6, 16]. LCM creates no chemical bonds to the target tissue so molecules in LCM-transferred cells are not degraded when compared to the original tissue slide [17]. Furthermore, LCM isolates cells via firm adherence to the cap, reducing tissue loss, where other microdissection techniques require the removal of the isolated cells with the help of a needle tip or a microcapillary [1].

The LCM technique is easily documented via a database program able to record images of both captured cells and residual tissue before and after microdissection. This diagnostic record is critical for maintaining an accurate record of each dissection, and for correlating histopathology with subsequent molecular analysis [6, 16].

A final, critical advantage of LCM is its application to formalin-fixed paraffin-embedded (FFPE) material, one of the most widely practiced methods for clinical sample preservation and archiving. Recent discoveries show promising advances in the use of FFPE tissues with LCM and subsequent molecular analysis. Collections of FFPE tissues comprise an invaluable resource for retrospective molecular studies of diseased tissues, including translational studies of cancer development [7, 14].

5.2 Disadvantages

The few limitations of LCM mostly reflect the difficulties of microdissection in general [1]. Cell identification is performed in conjunction with a pathologist, and is based upon the morphological characteristics of the cells of interest [11]. However, sections for microdissection are dehydrated and kept without a coverslip, making visualization of certain samples difficult due to decreased cellular detail [6, 18]. This sometimes makes precise dissection of cells from complex tissues very difficult. However, this problem can be circumvented by special stains, in particular immunohistochemical stains, which help highlight cell populations to be isolated or

avoided [1]. Unfortunately, standard immunohistochemical staining protocols require several hours, which can lead to further degradation of RNA and protein by RNases and proteinases respectively [1, 2, 6]. Fixation, dehydration, and staining of tissue sections also makes "live-cell analysis" [18] impossible.

Another problem occasionally encountered in LCM is failure to remove selected cells from the slide [1]. This can result from lack of adherence of the cells to the EVA membrane, usually because of incomplete dehydration or a laser setting that is too low for complete permeation of the melted polymer into the section [1, 6]. On the other hand, increased adherence of the section to the slide can prevent the removal of the targeted cells [1]. As a result, isolation of large numbers of cells (e.g., for protein analysis) from many sections can require considerable time [2, 18].

Older machines face problems related to a minimum laser spot size of 7.5 μm, which imposes restrictions on the precision of LCM recovery and makes it difficult to isolate cells of interest without contamination. The more recent generation of LCM machines, capable of dissecting cells at single-cell level, have overcome these limitations [1].

6 Clinical Applications

6.1 An Overview

Laser capture microdissection has significantly enhanced the molecular analysis of pathological processes as it offers a simple and efficient technique for procuring a homogeneous population of cells from their native tissue via direct microscopic visualization. Laser capture microdissection makes it possible to analyze cellular function between neighboring, intermingling, and morphologically identifiable cells within complex tissues and organs [17]. Overall, LCM is applicable to molecular profiling of tissue in normal and disease states; this includes correlations of cellular molecular signatures within specific cell populations and the comparison of different cellular elements within a single-tissue microenvironment [11].

Laser capture microdissection-based molecular analysis is being used in many fields of research, including the study of normal cell biology, as well as in vivo genomic and proteomic states such as the profiling of cultured intervertebral disc cells, molecular analysis of skeletal cell differentiation, and gene expression in testicular cell populations [16, 19–23]. Other studies focusing on the molecular analysis of histopathological lesions and disease processes include: mapping genetic alterations associated with the progression of pre-malignant cancer lesions (breast cancer and their lymph node metastases, ovarian cancer and prostate cancer); analyses of gene expression patterns in multiple sclerosis, atherosclerosis and Alzheimer's disease plaques; diagnosis of infectious micro-organisms,

and the analysis of genetic abnormalities in utero from selected fetal cells in maternal fluids [1, 2, 9, 16].

Laser capture microdissection is currently being used in the Cancer Genome Anatomy Program (CGAP) and exemplifies the molecular advances that LCM offers, as it allows researchers to catalog genes that are expressed in human tissue as normal cells undergo pre-malignant changes and further develop into invasive and metastatic cancer. Changes in expressed genes or alterations in cellular DNA corresponding to a specific disease state can be compared within or between individual patients, as a large number of microdissected cDNA libraries (produced from microdissected normal and pre-malignant tissue RNA) have been produced and published on the CGAP web page. This catalog of gene expression patterns has the potential to provide clues to etiology and, hopefully, contribute to early diagnostic detection and more accurate diagnosis of disease, followed by therapies tailored to individual patients [11, 16, 17].

Laser capture microdissection has been applied to genomic analyses such as studies of X-chromosome inactivation patterns to assess clonality, promoter hypermethylation, restriction fragment length polymorphisms (RFLP), and single-strand conformation polymorphism (SSCP) analysis for the assessment of mutations in critical genes such as p53 and *K-RAS*.

Novel uses include cancer chemoprevention, biomarker discovery, and live and rare cell isolation. Laser capture microdissection has been used for biomarker discovery in various human tissue types and organ systems. In these studies, LCM is used in combination with DNA transcriptome profiling to identify differentially expressed genes [24]. Intermediate endpoint biomarkers (IEB), used to monitor the success of chemoprevention, have been successfully developed for prostate cancer, cervical carcinoma, and adenomas for colorectal cancer [24]. Finally, LCM has been applied to the study of live and rare cell populations. Remarkably, LCM has no influence on the viability, metabolism, and proliferation rate of isolated living cells where even an entire living organism (such as the nematode *Caenorhabditis elegans*) can be successfully transferred without compromising the biological composition or viability of the organism. Live cell LCM isolation equipment is available from several manufacturers [25]. Finally, LCM is being used to isolate rare cells. In this rapidly developing method, rarely occurring cells are identified with automated scanning software, immediately followed by computer-controlled LCM [25].

6.2 DNA Analysis

Microdissection is now an established technique used to collect homogeneous cell populations for the analysis of genetic alterations at the DNA level [1]. With the advent of efficient analytical methods for small amounts of biological material, LCM is applied in pathological diagnosis, classification, and treatment of tumors. It

even plays a major role in gene mutation studies where a homogenous tumor cell population is necessary for accurate genomic analysis [1].

6.3 RNA Analysis

Laser capture microdissection offers several other advantages for mRNA analysis as compared with other laboratory techniques such as mRNA in situ hybridization or immunohistochemistry. Microdissection of purified cells, in combination with methods such as real-time quantitative RT-PCR, allows for a precise determination of cell-specific gene expression [25]. Furthermore, LCM is an efficient technique that allows sampling of large numbers of cells without significant RNA degradation where tissue dehydration may even inhibit the activity of tissue RNases, thereby maintaining the tissue integrity during specimen handling and preparation [1].

Gene expression analysis is critical in uncovering the patterns related to neoplastic transformation; however, the simultaneous detection of multiple different messages is preferable over the examination of single or few expressed genes. Therefore, microdissected cells are used in conjunction with cDNA array hybridization or serial analysis of gene expression to reveal the differences in gene expression profiles of normal and neoplastic cells, or to show altered gene expression patterns at various stages of cancer progression. Laser capture microdissection is also an essential tool in this process, as mRNA from microdissected lesions is subsequently used as the precursor to creating cDNA and expression libraries from purified cell populations [1].

6.4 LCM and "Omics"

Recent advances have resulted in the generation of several new "omics" technologies that are being increasingly used to better understand multiple facets of the disease process such as biology, etiopathogenesis, and genetic drivers. Briefly, these technologies include genomics, proteomics, transcriptomics, ionomics, and metabolomics. One of the main differences that these technologies offer is they are discovery- rather than hypothesis-driven.

Genomics relates to the analysis of the complete genome in an effort to gain a better understanding of the function of individual genes. *Proteomics* aims to establish the complete set of proteins or the "proteome" that are important in normal cellular physiology. *Transcriptomics* relates to the analyses of all messenger RNA molecules in one cell or a population of cells. It differs from the exome in that it includes only those RNA molecules found in a specified cell population, and usually includes the amount or concentration of each RNA molecule in addition to the molecular identities. *Metabolomics* is the scientific study of chemical processes involving metabolites. Specifically, metabolomics is the "systematic study of the unique chemical fingerprints that specific cellular processes leave behind," the study of their small-molecule metabolite profiles. The metabolome represents the collection of all metabolites in

a biological cell, tissue, organ, or organism, which are the end products of cellular processes. mRNA gene expression data and proteomic analyses reveal the set of gene products being produced in the cell, data that represents one aspect of cellular function. Conversely, metabolic profiling can give an instantaneous snapshot of the physiology of that cell. One of the challenges of systems biology and functional genomics is to integrate proteomic, trans criptomic, and metabolomic information to provide a better understanding of cellular biology.

6.5 LCM and Proteomics

The normal proteome is compared to a disease state proteome such as cancer using a variety of analyses including western blotting, high-resolution two-dimensional polyacrylamide gel electrophoresis (2-D PAGE), and mass spectrometry and peptide sequencing. Proteomics is a complementary approach to gene expression studies and provides supplementary information not obtained through genome or transcriptome analysis [24, 25].

Deciphering alterations in proteomic profiles using LCM techniques offers the advantage of studying physiological relationships unique to protein analysis, thereby offering the potential to identify novel diagnostic and therapeutic targets. In one study, a simple yet efficient workflow for routine liquid chromatography tandem mass spectrophotometry analysis of LCM Catapulting isolates resulted in highly efficient protein recovery [26]. To further demonstrate the capability of this approach with human tissues, the authors then analyzed punch biopsies from normal skin and chronic would keratinocytes and found that LCM keratinocyte captures yielded results with excellent reproducibility. Using LCM techniques to examine solid tumor heterogeneity, Johann et al. found that the use of LCM enabled the capture of homogenous regions of cells exhibiting uniform histology leading them to conclude that with continued optimization, this approach may lead to an improved understanding of tumor heterogeneity and serve as an efficient and reliable platform for solid tumor biomarker discovery [27].

6.6 LCM and Genomics

Loss of heterozygosity (LOH) analysis has been pivotal in cancer research for mapping of tumor suppressor genes, localization of putative chromosomal “hot spots,” as well as the study of sequential genetic changes in pre-neoplastic lesions. Microdissection has become a key technique used in LOH studies, since pure populations of tumor cells are necessary, and contamination by even a few unwanted cells may result in inappropriate amplification (via PCR) of the “lost” second allele present in non-cancer tissue. The use of LCM appears to have had a significant impact in the application of LOH analysis.

Other genomic analysis that have been performed from LCM samples for analyses of mutations in critical genes or to identify genes that are crucial to the etiopathogenesis of the disease being

studied include analysis of patterns of X-chromosome inactivation to assess clonality, restriction fragment length polymorphism (RFLP), and single-strand polymorphism (SSCP). Examples include, but are not limited to, the following: microdissection of tumors resulting in the identification of genes involved in multiple endocrine neoplasia type I, identification of genomic aberrations in hepatocellular carcinoma, and the demonstration of intratumoral heterogeneity in p53 mutations in select malignancies [28]. LCM is a promising tool for comparative genomic hybridization since it allows for the rapid sampling of large numbers of purified cells from heterogeneous tissues.

6.7 LCM and Transcriptomics

By providing a fast and dependable method of procuring cells from tissues, LCM has expanded the growing potential of sequencing to carry out transcriptomics as it enables usage of this technology at the single-cell level. The use of LCM enables the collection of an exact determinable number of purified cells under controlled conditions. Thus, combining it with other methodologies such as real-time quantitative PCR allows for a more precise determination of cell-specific genes [29]. Examples include studies demonstrating that selective microdissection of blood vessels; high-density microarray analysis, quantitative PCR-based validation of microarray data as well as immunohistochemical analysis can all be performed on no more than a 3 mm punch biopsy [30]. The utility of combining LCM and cDNA microarray hybridization was established by a study demonstrating reproducible differences in gene expression between small and large ganglia by the capture of a 1000 cells using LCM [31]. Similarly, alterations in gene expression at various stages of breast cancer progression have been shown by combining LCM and cDNA with real-time quantitative PCR. The combination of LCM and next-generation sequencing is one way of resolving the entire transcriptome of specific cell types.

6.8 Single-Cell Analysis

Laser capture microdissection has been applied to the isolation of single cells for the analysis of specific targets such as the identification of point mutations in oncogenes such as *RAS* and the amplification of expressed gene sequences by RT-PCR. Additionally, microdissected single cells can be used as a template for whole genome amplification, the generation of expression libraries, or probes for expression profiling with cDNA arrays [1].

Saurez-Quian et al. have modified the LCM protocol specifically for single-cell capturing. In this technique a cylinder covered with EVA polymer membrane has replaced the large cap surface. This decreases the contact area with the tissue and increases the accuracy of procuring a homogenous cell population [1].

6.9 Specific Diagnostic Applications

6.9.1 Tumors

The identification of genetic mutations is paramount in the pathological diagnosis, classification, and treatment of tumors. Loss of heterozygosity studies performed via microdissection has shown that the frequencies of genetic alterations have been largely under-estimated such that there may even be heterogeneity present within a single tumor where some genetic changes occur early in tumorigenesis [24].

Furthermore, LCM has been applied to the study of protein alterations in pre-neoplastic lesions and their tumor counterparts in an effort to elucidate novel tumor-specific alterations in peptide products of cancer cells. From these proteomic studies, distinct protein expression patterns have successfully classified normal, pre-malignant, and malignant cancer cells collected using LCM from human tissues [24]. Recently, it has become possible to use smaller samples of cells (not more than 20–100 dissected cells per PCR) obtained via microdissection, allowing for a more refined study of pre-neoplastic lesions in addition to neoplastic lesions. This has been made possible using a combination of microdissection with primer extension preamplification and whole genome amplification techniques, thereby opening a whole new frontier in cancer research [24].

6.9.2 Clonality Studies

Assessing clonality via DNA analyses using LCM has played an instrumental role in identifying the multiple endocrine neoplasia type 1 gene (MEN1), and will hopefully uncover the genetic basis underlying other cancer types. In the case of MEN1, LOH analysis of 200 microdissected endocrine tumors narrowed the interval of the genetic aberration to 300 kb. This LOH information from LCM analysis was used in conjunction with haplotyping and newly identified polymorphic markers, and led to the identification of a new tumor suppressor gene responsible for MEN1 [1, 24].

Laser capture microdissection, in conjunction with DNA analyses, has the ability to distinguish the presence of two clonal populations in the same tumor site. Fend et al. have demonstrated this in malignant non-Hodgkin's lymphoma, where two phenotypically and morphologically distinct cell populations were present in the same tumor. In this study, LCM was used to procure homogenous samples of the two populations from immunostained slides. Subsequent sequencing of rearranged immunoglobulin genes confirmed the presence of two unrelated clones in all the cases. LCM played a pivotal role in this study, as PCR analysis of DNA obtained from whole sections was not able to detect the biclonal composition of the tumors [1].

6.9.3 Clinical Applications of LCM in Dermatopathology

Laser capture microdissection has broadened the role of dermatopathology in molecular diagnosis and has greatly enhanced the understanding of the pathogenesis of inherited skin diseases [9, 32, 33].

The examination of precancerous lesions by LCM has been applied to the study of melanomagenesis, as it is widely believed that benign nevi undergo genetic alterations that progressively lead to melanoma development. Laser capture microdissection is used to assess the incidence of genetic gains and losses in tumors and pre-neoplastic lesions, and in doing so, has the potential to uncover the molecular events associated with the transformation of banal nevi into malignant melanoma formation [34–36].

6.10 Nevi Versus Melanoma

From a histopathological perspective, melanoma development is tracked by a series of melanocyte transitions from easily characterized precursors. However, from a genetic perspective, these transitions are poorly understood [36]. Laser capture microdissection therefore has the potential to shed light on the genetic profiles of melanocytes as they undergo these morphological transitions, hopefully uncovering the molecular events that lead to melanomagenesis.

Using LCM to dissect distinct populations of nevic aggregates in association with melanoma, we have been able to show that banal nevic aggregates might serve as precursor lesions [37].

6.11 Clonality in Cutaneous T-Cell Lymphoma

Analysis of T-cell gene rearrangement in cutaneous T-cell lymphoma (CTCL) has led to the discovery that the earliest manifestation of CTCL may be "clonal dermatitis." Clonal dermatitis is a chronic form of dermatitis that contains a dominant T-cell clone but does not show the typical histologic features diagnostic for CTCL. Significantly, approximately 25% of clonal dermatitis cases develop into CTCL within 5 years, where the same clone is present in both the clonal dermatitis and the CTCL lesions, indicating that the clonal dermatitis clone is a precursor to the CTCL. Laser capture microdissection is ideal for this type of study as the often-sparse lymphocytic infiltrate can be specifically captured. Furthermore, these studies allow unprecedented investigations into the molecular pathogenesis of CTCL, which will hopefully lead to early disease detection and help guide gene therapy [38].

Laser capture microdissection is also able to demonstrate genetically different clones or gene mutations limited to one specific neoplastic population. This has been an important tool in cutaneous lymphoma lesions containing a mixed B- and T-cell population. Using microdissection followed by genotypic analysis, Gallardo et al. established that the lesion of interest in the case study was cutaneous B-cell lymphoma with a dual B- and T-cell genotype. Conventional methods were not able to make this distinction, therefore illustrating the usefulness of LCM in clinical diagnostics [39].

6.12 Infectious Diseases

Laser capture microdissection allows for the isolation of pure cell populations which can be screened through PCR for infectious agents depending on the clinical and histological suspicion. Laser

capture microdissection also plays an important role in routine histopathologic diagnostics and has been applied to the diagnosis of infectious diseases such as borreliosis, herpes simplex virus (HSV) infection, herpes zoster, Epstein-Barr virus infection, *Myobacterium tuberculosis*, and many others [5, 40].

6.13 Conclusions

Tissue-based laser microdissection is a powerful technique, which combines morphology, histochemistry, and sophisticated downstream molecular analysis. High speed, easy handling, and good control and documentation of dissected tissue make LCM an ideal tool for the rapid collection of larger amounts of tissue. Further technological advances such as touch-screen cell annotation, automated cell microdissection, and cell recognition software are leading to the next-generation machines with enhanced microdissection capabilities. The ability of LCM to visualize and capture specific populations of cells has made LCM an important diagnostic tool, not just in dermatopathology.

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Chapter 2

Laser Microdissection-Based Microproteomics of Formalin-Fixed and Paraffin-Embedded (FFPE) Tissues

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Abstract

Laser microdissection-based proteomics on formalin-fixed and paraffin-embedded tissues is usually performed from relatively large tissue areas or pools of multiple tissue pieces. However, several molecular pathology studies require working on very limited amounts of tissue. This is for example the case when very early cancer lesions have to be handled. Hereby, we present a method for the processing of very small pieces of formalin-fixed and paraffin-embedded tissues for proteomic purposes. This approach is designed in order to avoid sample loss during technical processing and to optimize the digestion of tissue areas containing as little as 2700 cells.

Key words Formalin-fixed and paraffin-embedded tissues, Histopathology, Cancer, Laser microdissection, Microproteomics

1 Introduction

Laser microdissection (LMD) or laser capture microdissection (LCM) is the gold standard for the accurate sampling of pure bulks of cells from tissue sections. The evolution of "omics" methods allowed depicting molecular contexts in tissues for a wide panel of biomolecules. Deoxyribonucleic acids (DNA), ribonucleic acids (RNA), metabolites, and proteins can be extracted and analyzed from regions of interest of tissues containing only one cell type [1]. Proteomics has greatly evolved within the last decades owing to instrumental improvements providing optimal separation and analysis of peptides from proteins extracted and digested from laser microdissected tissue samples [2, 3]. Liquid chromatography (LC) can be performed in two separation dimensions to reduce the high complexity of proteolytic products from tissues [4]. Possibly, prefractionation can also be performed before LC, adding a further separation dimension to reduce sample complexity [5]. Liquid chromatography is then usually coupled to a mass

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spectrometer in order to analyze eluted compounds. The field of mass spectrometry has also greatly evolved in terms of instrumentation. Improved mass resolution and analysis speed allow high-throughput detection and fragmentation of dozens of thousands of peptides in a few hours [6, 7]. Until recently, there was an unmet need of downscaling the amount of laser microdissected tissues for proteomic purposes. Formalin fixation combined with paraffin embedment (FFPE) tissue processing is widespread among pathology institutes [8]. FFPE tissue blocks indeed represent a gold mine for biomarker discovery studies. Recently, we developed a method for the proteomic analysis of cancerous FFPE tissue pieces containing 2700 cells [9]. The method consists in the direct digestion of formalin fixed proteins on the tissue piece itself. So far, it is the most efficient one in terms of proteolytic peptide yields, cost, and time. We present here the application of this approach for biomarker discovery purposes. The whole workflow has already successfully been applied to studies in the field of oncology [9, 10]. The biochemical procedure has also been tested for a proteomic investigation in the field of parasitology [11].

The approach also holds great promise for routine histopathological diagnosis.

2 Materials

2.1 Reagents and Materials

1. Polyethylene naphthalate (PEN) membrane glass slides for laser microdissection (Leica Microsystems, Wetzlar, Germany).
2. Laser microdissection device: Leica LMD 7000 (Leica Microsystems, Wetzlar, Germany).
3. Milli-Q H_2O.
4. Citric acid (CA) 50 mM.
5. CA 10 mM, pH 6.0.
6. NaOH 1 M.
7. Rapigest surfactant (Waters, Milford, MA) 0.1%.
8. NH_4HCO_3 (ammonium bicarbonate) 500 mM.
9. NH_4HCO_3 100 mM.
10. NH_4HCO_3 50 mM.
11. NH_4HCO_3 25 mM.
12. Dithiothreitol (DTT) 500 mM.
13. DTT 131 mM.
14. DTT 171.6 mM.
15. Iodoacetamide (IAM) 500 mM.
16. IAM 194.67 mM.

17. Aluminum foils.
18. Hydrochloric acid (HCl) 10 mM.
19. Trypsin gold, mass spectrometry grade (Promega, Fitchburg, WI) 1 μg/μL.
20. Trypsin gold, mass spectrometry grade (Promega, Fitchburg, WI) 0.5 μg/μL.
21. Acetonitrile (ACN) 100%.
22. Trifluoroacetic acid (TFA) 10%.
23. Microtubes 0.6 mL.
24. KIMWIPES disposable (Kimberly-Clark, Dallas, TX).
25. Centrifuge.
26. Thermoshaker.
27. Antistatic gun Zerostat 3 (Sigma-Aldrich, St. Louis, MO).
28. Binocular microscope.
29. Sonicator.
30. Cleaning paper.
31. Speed vacuum centrifuge.
32. ZipTip (ZT) cartridge C_{18} 2 μg (Millipore, Billerica, MA).
33. ZT C_{18} 5 μg (Millipore, Billerica, MA).
34. NH_4HCO_2 (ammonium hydroxide) 20 mM, pH 10.
35. Formic acid 0.1% in water.
36. Formic acid 0.1% in acetonitrile.
37. MassPREP Digestion Standard Mixtures (MPDS) Mix 1 and 2 (Waters, Milford, MA).
38. Speed vacuum (Thermo Scientific, Waltham, MA).
39. Ultra performance liquid chromatography (UPLC) Nanoacquity 2D (Waters, Milford, MA).
40. Reverse phase (RP) X-Bridge BEH C18 5 μm column (300 μm × 50 mm) (Waters, Milford, MA).
41. Trap column symmetry C18 5 μm (180 μm × 20 mm) (Waters, Milford, MA).
42. Analytical column BEH C18 1.7 μm (75 μm × 250 mm) (Waters, Milford, MA).
43. Orbitrap Mass spectrometer Q Exactive or Q Exactive plus (Thermo Fisher Scientific, Waltham, MA).

2.2 Citrate Buffer Solution

Citric acid stock solution (50 mM): weigh 0.96 g of citric acid and dissolve in 99.94 mL Milli-Q water.

NaOH 1 M: Weigh 40 g of NaOH and dissolve in 1 L Milli-Q water.

Citric acid 10 mM, pH 6.0: Transfer 20 mL of the stock solution (50 mM) to a 100 mL bottle, adjust to 50 mL with Milli-Q water. Adjust the pH to 6.0 by adding 1 M NaOH. Adjust the final volume of 100 mL with Milli-Q water.

2.3 NH_4HCO_3 (Ammonium Bicarbonate) Solutions

NH_4HCO_3 500 mM: Weigh 39.53 g of NH_4HCO_3 and dilute in 1 L Milli-Q water. Store at 4 °C in a glass bottle.

NH_4HCO_3 100 mM: Dilute 5× the NH_4HCO_3 500 mM solution: NH_4HCO_3 500 mM/Milli-Q water 1:4. Store at 4 °C in a glass bottle.

NH_4HCO_3 50 mM: Dilute 10× the NH_4HCO_3 500 mM solution: NH_4HCO_3 500 mM/Milli-Q water 1:9. Store at 4 °C in a glass bottle.

NH_4HCO_3 25 mM: Dilute 20× the NH_4HCO_3 500 mM solution: NH_4HCO_3 500 mM/Milli-Q water 1:19. Store at 4 °C in a glass bottle.

2.4 DTT 500 mM

Weigh 0.7713 g of DTT. Solubilize with 10 mL of Milli-Q water. Aliquot in tubes with 100–500 µL and cover with aluminum foil. Freeze at −20 °C until use.

2.5 DTT 131 mM in 50 mM NH_4HCO_3

Mix 10 µL of DTT 500 mM with 9.1 µL of Milli-Q water and 19.1 µL of 100 mM NH_4HCO_3 in a 0.6 mL microtube. Cover with an aluminum foil.

2.6 DTT 171.6 mM in 50 mM NH_4HCO_3

Mix 10 µL of DTT 500 mM with 4.57 µL Milli-Q water and 14.57 µL of 100 mM NH_4HCO_3 in a 0.6 mL microtube. Cover with an aluminum foil.

2.7 IAM 500 mM

Weigh 1.85 g of IAM. Solubilize with 20 mL Milli-Q water. Aliquot tubes with 100–500 µL. Freeze at −20 °C until use.

2.8 IAM 194.67 mM in 50 mM NH_4HCO_3

Mix 20 µL of IAM 500 mM with 5.68 µL Milli-Q water and 25.68 µL of 100 mM NH_4HCO_3 in a 0.6 mL microtube. Cover with an aluminum foil.

2.9 HCl 10 mM

HCl stock solution (1 M): Commercial solutions of HCl are usually 12 M. Dilute 12× the 12 M HCl solution with Milli-Q water to obtain a 1 M stock solution: HCl 12 M/Milli-Q water 1:11.

HCl 10 mM: Dilute 100× the 1 M HCl solution with Milli-Q water: HCl 1 M/Milli-Q water 1:99.

2.10 Trypsin Stock Solution (1 µg/µL)

Reconstitute 100 µg of lyophilized trypsin in 100 µL of 10 mM HCl.

Aliquot in tubes with 1, 2, or 3 µL depending on further use, and store at −20 °C until use.

2.11 Trypsin 0.5 μg/μL Dilute 2× a trypsin stock solution (1 μg/μL) in 25 mM NH_4HCO_3. Depending on the number of sample to process, the amount of stock trypsin solution should be adapted.

2.12 Rapigest SF Surfactant 0.1% Reconstitute one vial (1 mg) in 1 mL NH_4HCO_3 50 mM. Aliquot in tubes with 100 μL and store at −20 °C before use.

2.13 TFA 10% Mix 10 mL of TFA with 90 mL of Milli-Q water. Store at 4 °C in a glass bottle.

2.14 Zip-Tip (ZT) Solutions

ZT Solution 1 (ACN + 0.1% TFA): Mix 999 μL ACN with 1 μL TFA in a tube.

ZT Solution 2 (ACN/H_2O 1:1 + 0.1% TFA): Mix 499.5 μL ACN with 499.5 μL Milli-Q H_2O and 1 μL TFA in a tube.

ZT Solution 3 (H_2O + 0.1% TFA): Mix 999 μL H_2O with 1 μL TFA in a tube.

Larger volumes can also be prepared and stored in glass bottles at 4 °C.

2.15 20 mM NH_4HCO_2 (ammonium formate) solution pH 10

200 mM ammonium formate pH 10 stock solution: NH_4OH (ammonium hydroxide) commercial solutions are usually 25%. For 1 L of solution, use a 1 L graduated cylinder and place 850–900 mL of Milli-Q H_2O. Add 15.4 mL of NH_4OH 25%. Mix well. Add 1.62 mL HCO_2H (formic acid). Mix well. Adjust the pH to 10 with NH_4OH 25% or formic acid. Adjust the volume to 1 L with Milli-Q H_2O. Store in a glass bottle at 4 °C.

20 mM ammonium formate pH 10: for 1 L of solution, mix 100 mL of 200 mM ammonium formate pH 10 stock solution with 900 mL of Milli-Q H_2O. Check the pH. Store in glass bottles at 4 °C.

2.16 0.1% formic acid in water Mix 1 mL of formic acid with 999 mL of Milli-Q H_2O.

2.17 0.1% formic acid in acetonitrile Mix 1 mL of formic acid with 999 mL of ACN.

3 Methods

3.1 Tissue Sectioning, Cell Counting, and Microdissection

1. After surgery, immerse the tissue overnight in 10% formalin, dehydrate and embed in paraffin using well-known procedures widely used among institutes of pathology [12]. Cut 5 μm-thick sections and deposit on LMD membrane slides for LMD

and histological glass slides for hematoxylin and eosin staining (HE). From the HE stained section, count an equivalent number of cells in tissue(s) region(s) of interest between samples. Several software products exist for cell counting from tissues such as QuPath (https://qupath.github.io/). Some tools such as Cytomine (http://www.cytomine.be/) [13] also allow sharing online tissue annotations and comments of the tissues between different institutions.

2. Perform microdissection: we used the Leica LMD 7000 (Leica Microsystems, Wetzlar, Germany) with the following laser settings: laser wavelength: 349 nm, pulse energy: 52 J, numerical aperture: 15, specimen balance: 14, laser head current: 100%, pulse frequency: 174 Hz, focus offset: 50. The settings should be adapted to the tissue type.

3.2 Sample Preprocessing

This step consists in collecting the tissue pieces from the cap to the bottom of the tubes to improve monitoring during processing (*see* **Notes 1, 2** and **3**).

1. During laser microdissection, the tissue pieces are collected in tube caps. Store the tubes at 4 °C before use, in a clean environment.
2. Use the antistatic gun to get rid of static electricity during sample preprocessing.
3. Centrifuge the tube at 15,000 × *g* during 5 min at room temperature (RT) and dispose the tubes in a clean holder.
4. Check for the presence of the tissue piece(s) at the bottom of the tubes using a binocular microscope. In Fig. 1 is illustrated the visualization of a LMD breast cancer tissue piece observed under the binocular microscope.

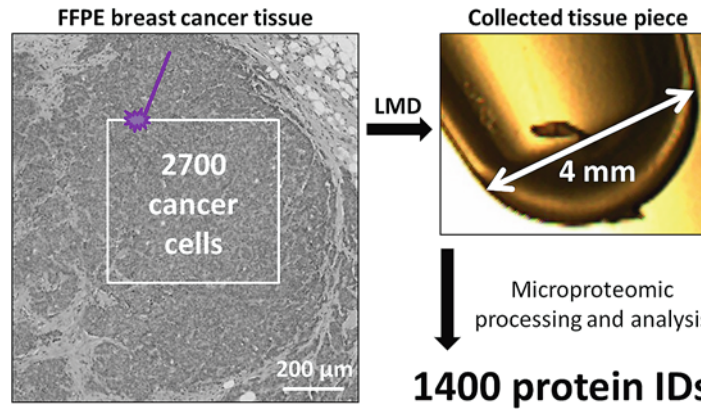

Fig. 1 Illustration of the collection of a tissue piece by LMD and the verification of its presence at the bottom of the tube through a binocular microscope. From a breast cancer tissue piece containing 2700 cells, it was possible to retrieve 1400 protein identifications [9] using the workflow described below. Adapted from [9]

3.3 Antigen Retrieval

This step consists in getting rid of the methylene bridges linking proteins induced by formalin fixation.

1. Gently add 20 µL of 10 mM pH 6.0 citrate buffer on the tissue piece at the bottom of the tube without touching it with the tip.
2. Centrifuge at 15,000 × *g* for 5 min at RT. If it is still visible, ensure yourself that the tissue is properly immersed in citric acid.
3. Sonicate the immersed tissue pieces for 10 min in a holder that avoids overheating of the tubes. This step allows removing air bubbles that may stay at the surface of the tissue.
4. Incubate in a thermoshaker at 99 °C for 1 h at 800 rpm. Quickly spin the tubes every 10 min in order to collect the condensed water from the cap to the bottom of the tubes.
5. Collect the tubes in a clean holder and let these cool at RT.
6. Add 2.2 µL of Rapigest 0.1% in the samples to obtain a final 0.01% concentration and quickly spin.
7. Shake at 800 rpm for 10 min at RT.
8. Add 2 µL of 500 mM NH_4HCO_3 to all the samples. Shake 10 min at 800 rpm, quickly spin and check that the pH of the samples is 7.

3.4 Reduction-Alkylation-Reduction

This step consists in getting rid of disulfide bridges still existing within proteins after antigen retrieval (*see* **Notes 4** and **5**).

1. Reduction (reduction of the disulfide bridges and liberation of the thiol groups): the samples have now a volume of 24.2 µL. Add 2 µL of 131 mM DTT in 50 mM NH_4HCO_3 to obtain a final concentration of 10 mM DTT. Incubate the samples for 40 min at 56 °C while shaking at 800 rpm and quickly spin.
2. Alkylation (blocking thiol groups with IAM): the samples have now a volume of 26.2 µL. Let the samples cool down at RT and add 3 µL of 194.67 mM IAM in 50 mM NH_4HCO_3 to obtain a final concentration of 20 mM IAM. Incubate at RT for 30 min while shaking at 800 rpm and quickly spin.
3. Reduction (eliminate IAM in excess): The samples have a volume of 29.2 µL. Add 2 µL of 171.6 mM DTT in 50 mM NH_4HCO_3 to add a concentration of 11 mM DTT. Incubate 10 min at RT while shaking at 800 rpm and quickly spin.

3.5 Digestion

This step consists in digesting the proteins from the tissue piece (*see* **Notes 6** and **7**).

1. The samples have a volume of 31.2 µL. Add 4.26 µL of 0.5 µg/µL trypsin to obtain a final concentration of about 60 µg/mL.
2. Incubate at 37 °C overnight while shaking at 800 rpm and quickly spin.

3. The samples have a volume of 35.46 µL. Add 2.26 µL of 0.5 µg/µL trypsin to increase the concentration by about 30 µg/mL.
4. The samples have now a volume of 37.72 µL. Add 150.88 µL of ACN to obtain a solution of 80% ACN and quickly spin.
5. Incubate at 37 °C for 3 h while shaking at 800 rpm and quickly spin.
6. The samples have a volume of 188.6 µL. Stop digestion by adding 10 µL of TFA 10% to obtain a final concentration of about 0.5% TFA in the tube. Shake 10 min at 800 rpm, quickly spin and check the pH of the solution (pH < 3).
7. Incubate for 45 min at 37 °C while shaking at 800 rpm in order to cleave Rapigest.
8. Centrifuge the samples at 21,000 × *g* for 10 min, at 4 °C.
9. Collect the supernatant and transfer in new identified 0.6 mL tubes.
10. Evaporate the samples using the Speed Vacuum. Store the samples at 4 °C if necessary for 1 week maximum. If the storage time exceeds 1 week, store the samples at −20 °C.

3.6 Purification/ Desalting/ Concentration of the Samples

This step consists in purifying, desalting the sample and concentrate the proteolytic peptides by solid phase extraction (SPE).

1. Prepare the three solutions described in Subheading 2.14.
2. If the samples were dried, resuspend in 20 µL Milli-Q H_2O with 0.1% TFA (ZT solution 3). If the SPE is performed just after the digestion, transfer the necessary volume of sample in another 0.6 mL tube and adjust to 0.1% TFA, with maximum 5% of organic solvent, in an ideal volume of 20 µL. Depending on the expected amount of peptides resulting from the digestion process, use ZT C_{18} 5 µg or ZT C_{18} 2 µg (µZT).
3. Set the micropipette to 10 µL. Wash the ZT with solution 1: take and withdraw in the waste. Repeat three times.
4. Activate the ZT by taking solution 2. Withdraw in the waste. Repeat three times.
5. Equilibrate the ZT: take solution 3 and withdraw in the waste. Repeat three times.
6. Load the sample. Adjust the sample to the used ZT. If the expected concentration of peptides in the sample is too high (>5 µg when ZT 5 µg are used, >2 µg when ZT 2 µg are used), dilute with a 0.1% TFA solution. Pipet ten times to load the peptides to the ZT. If the sample is diluted or if the volume of sample is higher than 20 µL, pipet 25 times.
7. Wash the samples: take solution 3 and withdraw in the waste. Repeat five times.

8. Elute the sample: Take solution 2 and collect in a new microtube. The peptides are present in the solution in the new microtube. Repeat once. The proteolytic digest is then concentrated in 40 μL of ACN/ H_2O 1:1 and 0.1% TFA.
9. Evaporate the sample completely with the speed vacuum centrifuge.
10. Proceed to the subsequent LC-MS/MS analysis or store the sample at 4 °C for 1 week maximum, at −20 °C if the analysis cannot be performed within 1 week.

3.7 Two Dimensions NanoLC-Orbitrap Mass Spectrometry Analysis

1. Resuspend the samples in 10–11 μL of appropriate buffer for 2D LC-MS and MPDS Mix in order to get a final concentration of 50 fmol alcohol deshydrogenase (ADH) per volume of injection (9 μL). Use different MPDS Mix solutions (1 or 2) in samples of different types. For example: if two conditions are compared (A and B), use MPDS mix 1 for samples from condition A and MPDS mix 2 for samples from condition B. This will allow us to further control that the ratio of proteins from MPDS mixes 1 and 2 is the one expected.
2. 9 μL of the samples are injected in the 2D LC-MS system of choice (UPLC Nanoacquity 2D (Waters, Milford, MA) controlled by MassLynx in our case) coupled to a Q Exactive Plus, controlled by XCalibur. *See* **Notes 8** and **9**.
3. In our case, the samples are then injected in a two dimensions RP/RP system, with a first dimension in a high pH (pH 10) and a second dimension in a low pH (pH 3). The peptides are loaded on the high pH column (X-Bridge BEH C18 5 μm (300 μm × 50 mm)) at 2 μL/min (20 mM ammonium formate solution adjusted to pH 10) and three elution steps (15 min each) with the following percentages of ACN are realized: 13.3% (fraction 1), 19% (fraction 2), and 65% (fraction 3).
4. The eluate from the "high pH" column is then diluted ten times with acidified water before being loaded on the trap column (Symmetry C18 5 μm (180 μm × 20 mm)) and separated on the "low pH" analytical column (BEH C18 1.7 μm (75 μm × 250 mm)). The gradient on the low pH column is 140 min long with the following settings: flow rate of 250 nL/min, solvent A (0.1% formic acid in water), and solvent B (0.1% formic acid in acetonitrile) with a linear gradient as follows: 0 min, 99% A; 5 min, 93% A; 140 min, 65% A. Cleaning and re-equilibration steps then take place during the following 40 min (total run of 180 min).
5. The LC eluent is then directly electrosprayed from the analytical column at 2.1 kV voltage through the liquid junction of the nanospray source. The chromatography system was coupled to a Thermo Scientific Q Exactive Plus Hybrid quadrupole-

orbitrap mass spectrometer (Thermo Fisher Scientific, USA), programmed for data-dependent acquisition mode, with the following settings:

- Top 10 (Data Dependent Acquisition).
- Parameters for MS: mass range: m/z 400–1750, resolution: 70,000, AGC target: 1×10^{e6}, maximum injection time: 200 ms.
- Parameters for MS/MS: isolation window: m/z 2.0, stepped normalized collision energy (NCE): 21.2, 25, 28.8, Resolution: 17,500; AGC target: 1×10^{e5}, maximum injection time: 200 ms, underfill ratio: 1.0%, dynamic exclusion: 10 s.

3.8 Data Processing for Biomarker Discovery

We propose here a method for biomarker discovery using the well-known and widely used software Maxquant and Perseus (*see* **Note 10**). The present settings were designed for the comparison of two different conditions (for example two cancer types from the same organ such as squamous cell carcinoma of the lung vs. adenocarcinoma of the lung).

1. Label-Free Quantification (LFQ)

   Use the last version of Maxquant software. Load the samples from the two conditions to compare. At least five biological replicates per condition should be processed. For the identification, use Andromeda search engine with the last release of Uniprot human database for interrogation. Use the following parameters: N-ter acetylation, oxidation of methionines as variable modifications, carbamidomethylation of the cysteines as fixed modification. Set the maximum number of miscleavages at 2 and the minimal length for identification at 7 amino acids and at least two peptides required for identification, including one unique peptide. Check "LFQ" for data normalization. Set the maximum ratio count for LFQ at 2. Set the main search tolerance at 4.5 ppm. Set peptide spectrum match (PSM) and false discovery rate (FDR) at 0.01.

   For detailed explanations on the different parameters for MaxQuant and an informed choice of these for data processing setup, please refer to [14].

3.9 Statistical Analysis

Use the latest version of Perseus. Filter rows by removing "only identified by site" and "reverse" entries. Log_2 transform the intensities. Perform the categorical annotation by annotating samples from each condition with the same name. Add annotation to the samples with gene ontologies (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Filter rows based on valid values by keeping 4/5 values in at least one group. Perform a two samples *t*-test with the fold change of interest, preferentially 2 (S0 = 1), a FDR of 0.01 and 250 randomizations. Perform a

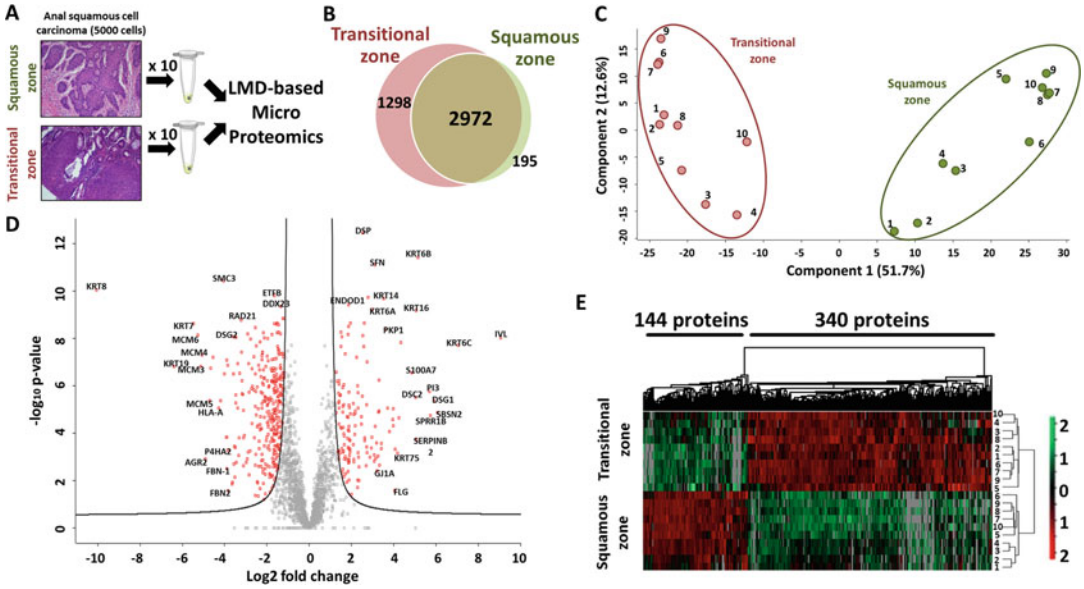

Fig. 2 Example of biomarker discovery study [10]. Ten biological cases from two types of anal carcinoma originating from different microanatomical regions of the anal canal were selected, each. The LMD-based microproteomics method was applied on tissue pieces containing 5000 cancer cells (**a**). A total of 4465 protein IDs were retrieved, including 2972 commune proteins between the two cancer types (**b**). The principal component analysis of the dataset clearly allowed distinguishing the samples from the two different cancer types, on a molecular basis (**c**). The *t*-test analysis allowed us to retrieve about 500 protein markers with at least two-fold variation between the two cancer types. These can be visualized within a Volcano plot. In this representation, every marker is highlighted in red (**d**). Hierarchical clustering of the dataset based on those 500 proteins also allowed deciphering the two types of cancers (**e**). Adapted from [10]

volcano plot with the same settings. Every significantly up or down-regulated protein stands upon the lateral lines. For PCA, filter the data from all the non-valid values and perform the PCA without category enrichment for components. For clustering, previously perform *Z*-score normalization. Clustering can be performed from the whole dataset of filtered data (for example *t*-test positive values). Euclidian distance and average linkage without *k*-mean should be used as parameters.

For detailed explanations on the different parameters for Perseus and an informed choice of these for statistical analysis, please refer to [15].

The results of a biomarker discovery study are illustrated in Fig. 2.

4 Notes

The overall workflow relies on a principle of on-tissue digestion. In this context, the laser microdissected tissue pieces have to remain in the tubes during the whole tissue processing, until the end of the

digestion steps. Several considerations have to be taken into account in order to avoid sample loss. Some other chemical parameters have to be adapted for the optimal digestion of the protein from the tissue samples. Finally, specific analytical settings are more adequate to the analysis of restricted amounts of proteolytic peptides from small tissues pieces.

1. The number of tissue regions to be microdissected should be reduced to the minimum in order to properly monitor the presence of the tissue piece(s) at the bottom of the tube after centrifugation.
2. The tubes should not be opened before centrifugation.
3. Under the binocular, the tissue pieces should not be confused with eventual scratches at the bottom of the tube that could be caused by the tubes holder during LMD.
4. Nothing should touch the tissue piece during the processing. Solutions should then be added by letting it flow on the boundaries of the tube.
5. The reactions have then to take place at the bottom of the tube and everything has to be set to restrict the reactions in this same exact location. Shaking is then used and not vortexing. By vortexing, the reaction buffer could indeed carry the tissue piece to the boundary of the tube where it could stay sticked.
6. The extraction process during this workflow is minimal and the digestion of the proteins occurs majorly on the tissue piece itself. In order to allow the optimal digestion of the proteins concentrated in such a limited volume, high concentrations of trypsin should be used. The digestion takes place in two steps. The first one occurs in 50 mM NH_4HCO_3 buffer and represents the "classical" digestion step. The second step occurs in 80% ACN and allows the digestion of the most hydrophobic proteins.
7. The digestion is enhanced with low concentrations of RG (0.01%). This detergent can be used during the digestion, without any clean-up procedure for its removal. A cleanup before digestion indeed leads to the loss of the tissue piece [9]. RG helps for the digestion of the most hydrophobic proteins. We observed that using low concentrations of RG allowed retrieving a higher number of peptide identifications [9].
8. The analytical procedure is adapted to weakly concentrated mixtures. The processing of small laser microdissected tissues pieces gives rise to low concentrations of peptides. It was proven before that the combination of long LC elution time and long injection time for MS/MS allows retrieving a high number of peptide/protein identifications.

9. Different combinations of instrumentation can be used for LC and MS. Today, MS data from many vendors are compatible to Maxquant processing. However, a large panel of statistical software solutions exists for data normalization and processing.
10. The method presented here is set up for biomarker discovery applications. In the near future, the approach is expected to have applications in routine histopathological diagnosis.

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Chapter 3

Laser Microdissection Workflow for Isolating Nucleic Acids from Fixed and Frozen Tissue Samples

Yelena G. Golubeva and Andrew C. Warner

Abstract

Laser Capture Microdissection has earned a permanent place among modern techniques connecting histology and molecular biology. Laser Capture Microdissection has become an invaluable tool in medical research as a means for collection of specific cell populations isolated from their environment. Such genomic sample enrichment dramatically increases the sensitivity and precision of downstream molecular assays used for biomarker discovery, monitoring disease onset and progression, and in the development of personalized medicine. The diversity of research targets (cancerous and precancerous lesions in clinical and animal research, cell pellets, rodent embryos, frozen tissues, archival repository slides, etc.) and scientific objectives present a challenge in establishing standard protocols for Laser Capture Microdissection. In the present chapter, we share our experiences in design and successful execution of numerous diverse microdissection projects, and provide considerations to be taken into account in planning a microdissection study. Our workflow and protocols are standardized for a wide range of animal and human tissues and adapted to downstream analysis platforms.

Key words Laser capture microdissection, UV laser, IR laser, LCM workflow, RNA, DNA, Molecular analysis, Frozen tissue, FFPE tissue, Large-scale LCM project, Molecular fixatives

1 Introduction

Scientific interest in genomic and proteomic analysis of specific cell populations lead to the development of Laser Capture Microdissection (**LCM**) [1–4], a powerful research tool that directly connects histology and molecular biology. During the past decade, genes have become the main focus of basic and clinical studies for cancer, Alzheimer disease, multiple sclerosis, immunodeficiency, and many others. Nucleic acids (RNA/DNA) and proteins became the targets of interest for discovery of genes and validation of their functions [5–8]. The molecular analysis of complex tissues is not reliable for finding subtle changes in molecular signatures and the correlation of cellular molecular signatures with specific cell populations [9–12]. LCM provides an enrichment of genomic sample which

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dramatically increases the sensitivity and precision of downstream molecular assays used for biomarker discovery, monitoring disease onset and progression, and in the development of personalized medicine. As a unique research tool in molecular pathology, LCM has been increasingly incorporated in core services of major research centers worldwide. The need for simultaneous delivery of quality histological material for pathology evaluation and intact material for subsequent genomic and proteomic studies imposes challenging conditions on traditional methods of tissue collection and slide preparation. The quality of isolated genomic material is critical for the accuracy of analysis, especially by high-throughput molecular techniques [13–16], which makes LCM a challenging technique. LCM, as a multistep technique, warrants a special set of skills and planning for each of the steps, with molecular integrity of the tissue in mind for all the steps of LCM workflow from tissue collection to retrieval of target molecules. LCM projects, requiring RNA retrieval, are especially challenging due to the unstable nature of RNA. Stability of RNA in different tissues varies significantly [17, 18], as does the morphological appearance of target cells on the dissecting screen [18–21] and section adherence to glass or membrane slides [18, 20–22]. A customized, target-dependent approach to all the steps of LCM (sample preparation, dissection and nucleic acid/protein purification, and analysis) has been considered the only reliable way to a successful LCM project [4, 18–20].

When we started LCM as a core service of a histopathology laboratory for a multidiscipline research community, we understood the necessity of developing an LCM workflow with reliable protocols applicable to a wide variety of samples and tissue types. Moreover, tailoring the dissection approach and processing of targeted molecules for the requirements of prospective downstream analysis, especially in large-scale LCM projects, warranted that pilot studies become a vital part of LCM workflow. We also concluded that it was advantageous to have an LCM instrument with infrared laser for single-cell type dissections, and, for laser cutting projects, an instrument with only a UV laser but excellent visualization on the LCM dissecting screen. This combination of LCM instruments proved to be crucial for bringing to life projects deemed unfeasible. This was especially so for working with archival formalin-fixed and paraffin-embedded (FFPE) samples.

In the present chapter, we concentrate on LCM workflow which incorporates digital slide imaging and web-based pathology annotations. This workflow, created for large-scale LCM studies, can be easily adjusted to the goals and scale of a particular project using presented protocols and approaches as building blocks within available resources.

Also, LCM workflow modifications are described for specific challenging LCM projects. With the emphasis on preservation of

tissue molecular integrity, we address the methodology of sample collection (LCM studies based on rodent animal models), and sample handling and processing (LCM studies based on biorepository sets of human samples). Our LCM slide preparation protocols were standardized either for frozen samples embedded in optimum cutting temperature medium (**OCT**) or paraffin-embedded samples, and are applicable for a wide variety of tissue types. Special attention is paid to the concept of a pilot study in LCM projects which secures the success of bringing together upstream process (LCM) with downstream analysis of molecules derived from LCM targets. The planning of pilot studies starts with the analysis of information pertaining to the project details and scale, projected downstream applications and available resources, with the prospective sample set in mind. Information from the pilot study allows adjustments to each step of LCM workflow which will be used for the main study. We provide the protocols for the use of molecular fixatives in animal studies to overcome the problem of poor morphology and molecular instability in frozen sections during LCM. Modifications of DNA and RNA extraction protocols from LCM samples to satisfy the type of tissue fixation and input requirements of the downstream analysis platform are also discussed in detail. We believe that our LCM workflow, protocols, and approaches, optimized for core LCM support on a wide variety of samples, will be valuable resource to scientists in different research areas, especially in cancer biology.

2 Materials

2.1 LCM Workflow: Sample Acquisition

1. Necropsy protocol.
2. Necropsy station with RNase-free tools.
3. Euthanasia chamber.
4. CO_2.
5. Razor blades.
6. RNase-AWAY™.
7. Nuclease-free water.
8. Kimwipes.
9. Tissue-Tek OCT Compound (Sakura Finetek USA).
10. Cryomolds and Adhesive Cryolabels.
11. 2-methylbutane (ThermoFisher Scientific).
12. Dry ice.
13. Wet ice.
14. Styrofoam box.
15. Resealable plastic bag.

16. RNase-free molecular fixatives: PAXgene, (PreAnalytiX), TheraLin (Grace Bio-Labs, Bend, OR), DSP (Lomant's Reagent, ThermoFisher Scientific) (*see* **Note 1**), STU™ Technology molecular fixative (Mt. Washington Scientific) (**STUMol**).
17. RNase-free containers for molecular fixatives (100 ml).
18. RNaseOut™ RNase inhibitor (Invitrogen) (**RNaseOut**).
19. Labeled tissue cassettes.
20. Plastic cutting board.
21. Orbital shaker.
22. Automated tissue processor.
23. Xylene.
24. 100% ethanol.
25. Isopropyl Alcohol.
26. Paraplast Plus (McCormick), molten.
27. Embedding center or hot and cold plate for embedding.
28. RNase-free forceps.
29. Liquid nitrogen.
30. Metal ladle.
31. Gauze.
32. Hammer.
33. RNase-free weigh boats.
34. Labeled cryo containers.
35. Timers.
36. Biosafety cabinet.
37. Embedding molds.
38. Tissue Stabilizer Concentrate (Qiagen).

2.2 LCM Workflow: Quality Control (QC) of Molecular Integrity in Acquired Tissues and Pathology Evaluation of Tissue Blocks for LCM Objectives

1. **Items 6–9**, **12–15**, **23–25**, **28**, and **35** from Subheading 2.1.
2. Tissue blocks from Subheading 2.1: fresh frozen OCT tissues, DSP-fixed OCT tissues, tissues fixed in molecular fixatives and embedded in paraffin (**FMFP**).
3. Cryostat.
4. Microtome.
5. Microtome blades.
6. Art brushes.
7. 1.5 ml nuclease-free micro-centrifuge tube for high G-force (VWR).
8. 1.8 ml nuclease-free micro-centrifuge tube with screw cap.
9. Glass Microscope slides, superfrost plus (charged) (GSS).

10. Adhesive slide labels.
11. RNeasy® Micro Kit, Qiagen.
12. PAXgene® Tissue miRNA Kit, PreAntalytiX® (**PAXgene**).
13. Linear polyacrylamide, GenElute™-LPA Sigma (**LPA**).
14. 2 β-mercaptoethanol (Sigma).
15. Protease inhibitors: Complete Protease Inhibitor Cocktail tablets (Roche).
16. 1,4-Dithiothreitol, Sigma (**DTT**).
17. Proteinase K, 20 mg/ml, ThermoFisher Scientific (**PK**).
18. RNase-free barrier pipette tips.
19. Vortexer.
20. NanoDrop™ Spectrophotometer (**NanoDrop**).
21. Agilent 2100 Bioanalyzer.
22. Agilent RNA 6000 Pico Chip (Agilent Technologies) (**Pico Chip**).
23. Agilent Bioanalyzer 2100 Expert software (Agilent Technologies, Santa Clara, CA).
24. Hematoxylin-2 (VWR).
25. Eosin Y (VWR).
26. Bluing reagent (VWR).
27. Plastic slide holder with staining container, 24 places.
28. Resin mounting medium.
29. Aperio® AT2 digital slide scanner, Leica Biosystems (**Aperio**).
30. Aperio ImageScope™ software.
31. Centrifuge with a 2.0 ml tube rotor.

2.3 LCM Workflow: Pilot Study

1. Two tissue blocks selected from study set in QC from Subheading 2.2 or archival FFPE tissue blocks with the largest annotated LCM target.
2. **Items 5–9**, **12–15**, **18**, **23–25**, **28**, **33**, and **35** from Subheading 2.1; **Items 3–31** from Subheading 2.2; **Items 3–21** from Subheading 2.4; **Items 3–11** from Subheading 2.5; **Items 3–6** from Subheading 2.6; **Items 3–11** from Subheading 2.7; **Item 3** from Subheading 2.8.

2.4 LCM Workflow: Preparation of LCM Slides for Main Study

1. **Items 5–9**, **12–15**, **23**, **24**, **28**, **33**, and **35** from Subheading 2.1; **items 3–6**, **10**, **11**, **15**, **18**, **19**, **24–28**, and **30** from Subheading 2.2.
2. Tissue blocks selected for LCM study from Subheading 2.2.
3. Metal frame membrane slides (MMI Molecular Machines & Industries).

4. Glass membrane slides (ThermoFisher Scientific).
5. RNase-free Glass Microscope slides (ThermoFisher Scientific).
6. The CryoJane® Tape-Transfer System (Leica Microsystems) (**CryoJane**).
7. CryoJane consumables: Adhesive Solutions, Transfer Tape, Adhesive glass slides, CryoJane Hand Roller.
8. Ice pan with solid ice (kept at −20 °C).
9. RNase-free water bath at +43 °C.
10. RA Lamb Five-Slide Mailer (ThermoFisher Scientific) (**Five-Slide Mailer**).
11. Ultraviolet light chamber at 352 nm.
12. Lab marker.
13. Sections mounted on membrane and CryoJane slides.
14. 50 and 14 ml conical polypropylene tubes.
15. Glacial acetic acid.
16. Acetone.
17. ProtectRNA™RNase inhibitor (Sigma) (**ProtectRNA**).
18. MethylGreen (Vector Laboratories).
19. Cresyl Violet Acetate (Sigma): 1% solution in 100% ethanol (**CVA**) (*see* **Note 2**).
20. Desiccator with desiccant.
21. mySPIN™ Mini Centrifuge (ThermoFisher Scientific).

2.5 LCM Workflow: Dissection of LCM Slides

1. **Items 5–8**, **12–14**, and **35** from Subheading 2.1; **item 30** from Subheading 2.2.
2. Stained LCM slides from Subheading 2.4.
3. MMI CellCut (MMI Molecular Machines & Industries).
4. MMI IsolationCap® (MMI Cap) (1, 5, 0.5 and 0.2 ml) (**MMI Cap**).
5. RNase-free Glass Microscope slides (ThermoFisher Scientific) for metal frame membrane slide support.
6. Arcturus® XT LCM System (ThermoFisher Scientific) (**Arcturus XT**).
7. CapSure® HS Cap (**HS Cap**) and CapSure® Macro Cap (**Macro Cap**).
8. Computer with wide monitor (*see* **Note 3**) (**Reference Computer**).
9. Conventional dissecting microscope.
10. RNase-free Inox #5 forceps (Roboz Surgical Instrument).
11. Scotch Tape (*see* **Note 4**).

2.6 LCM Workflow: Lysate Preparation from LCM Targets

1. **Items 5–8**, **12–15**, **24**, and **35** from Subheading 2.1; **items 18** and **19**, from Subheading 2.2; **items 10** and **11** from Subheading 2.4; **items 10** and **11** from Subheading 2.5.
2. Dissected targets on collection caps or dissected slides from Subheading 2.5.
3. Lysis buffer of choice, prepared or ready for use from isolation kits (**items 3–9** from Subheading 2.7).
4. Nuclease-free 1.5 ml centrifuge tubes, 200 µl PCR tubes, 0.5 and 1.8 ml centrifuge screw cap tubes.
5. Thermo Scientific™ Thermal mixer (ThermoFisher Scientific).
6. Fisherbrand™ Cryo/Freezer Box (ThermoFisher Scientific).

2.7 LCM Workflow: Retrieval of Target Molecules from LCM Lysates

1. **Items 6–8**, **12–15**, **24**, **25**, and **35** from Subheading 2.1; **items 13–20**, and **31** from Subheading 2.2; **item 13** from Subheading 2.4; **items 5** and **6** from Subheading 2.6.
2. LCM lysates from Subheading 2.6.
3. AllPrep® DNA/RNA Micro Kit (Qiagen).
4. miRNeasy® FFPE Kit (Qiagen) (**FFPE Kit**).
5. RNeasy® Micro Kit, Qiagen.
6. PAXgene® Tissue miRNA Kit, PreAntalytiX®.
7. Arcturus® PicoPure® DNA extraction Kit (ThermoFisher Scientific).
8. Animal Tissue DNA Extraction Kit, Autogen.
9. QIAamp DNA Micro Kit, Qiagen.
10. Savant DNA120 SpeedVac (ThermoFisher Scientific).
11. 1.8 ml centrifuge screw cap tubes.

2.8 LCM Workflow: Quality Control of RNA and DNA Purified from LCM Targets

1. **Items 6–8**, **12–14**, and **35** from Subheading 2.1; **items 18**, **19**, **21–23**, and **31** from Subheading 2.2; **item 13** from Subheading 2.4; **item 5** from Subheading 2.6.
2. Purified RNA and DNA from LCM targets from Subheading 2.7.
3. Quantifiler® Human DNA Quantification Kit, (ThermoFisher Scientific) (**Quantifiler**).
4. RNase-free 200 µl PCR tubes.

2.9 Selected LCM Studies: Large-Scale Study on Archival FFPE Sections Mounted on Glass Slides

1. **Items 6–8**, **12–14**, **24**, and **35** from Subheading 2.1; **items 2–31** from Subheading 2.2, **items2–21** from Subheading 2.4, **items 5** and **6** from Subheading 2.6, **items 3–5** and **7–11** from Subheading 2.7.
2. Archival slides with FFPE sections.
3. Compressed air Fisherbrand® Super Friendly air it™ (ThermoFisher Scientific).

2.10 Selected LCM Studies: Dissection of Early Rodent Embryos for Genotyping

1. **Items 5–8**, **13–15**, **24**, and **35** from Subheading 2.1; **items 18**, **19** and **24–31** from Subheading 2.2, **items 3–12** and **19–21** from Subheading 2.4; **items 3–11** from Subheading 2.5, **items 5** and **6** from Subheading 2.6, **items 3**, **7**, and **11** from Subheading 2.7.
2. Slide with mounted FFPE/frozen OCT sections of embryo sample.
3. Nuclease-free 0.5 ml centrifuge screw cap tubes.
4. Syringe needle, 27 g.
5. Ambidextrous Pinning Forceps and 0.15 mm minuten pins (BioQuip).
6. Incubation oven (up to +65 °C).

2.11 Selected LCM Studies: Laser Capture of H2AX Positive Cells on FFPE Sections of Human Tonsil for RNA and DNA Retrieval

1. **Items 5–8**, **12**, **14**, **18**, **23–25**, **28**, **33**, and **35** from Subheading 2.1; **Items 4–10**, **13**, **14**, **17–19**, and **20–31** from Subheading 2.2; **Items 8**, **9–12**, **14**, **20**, and **21** from Subheading 2.4; **Items 3** and **6–11** from Subheading 2.5; **Item 5** from Subheading 2.6; **Items 4** and **8** from Subheading 2.7; **Item 3** from Subheading 2.8.
2. Standard IHC protocol for H2AX marker, primary and secondary antibody, protocol reagents and equipment.
3. FFPE tissue sections mounted on glass slides.
4. Slide Humidity Incubation Box.
5. Trypsin.
6. Phosphate-buffered saline (**PBS**).
7. Arcturus PixCell IIe (**PixCell IIe**).

2.12 Selected LCM Studies: Laser Capture of Insulin Positive Pancreatic Islets on Frozen Repository Samples of Human Pancreas

1. **Items 5–8**, **12**, **14**, **18**, **23–25**, **28**, **33**, and **35** from Subheading 2.1; **Items 4–10**, **13**, **14**, **17–19**, and **20–31** from Subheading 2.2; **Items 8**, **9–12**, **14**, **20**, and **21** from Subheading 2.4; **Items 3** and **6–11** from Subheading 2.5; **Item 5** from Subheading 2.7; **Items 4** and **6** from Subheading 2.11.
2. Standard IHC protocol for insulin marker, primary and secondary antibody, protocol reagents and equipment.
3. Repository glass slides with frozen sections.
4. 20 mM Tris Buffer (pH 8).
5. Arcturus PixCell IIe.

2.13 Selected LCM Studies: Laser Capture of Tyrosine Hydroxylase (TH) Positive Neurons on Frozen Sections of Mouse Brain

1. **Items** 1–15,17,18,21 and 28 from Subheading 2.1; **Items 3**, **5–11**, **13**, **14**, **16**, **18–23**, **25**, and **27–31** from Subheading 2.2; **Items 4**, **14**, **15**, **20**, and **21** from Subheading 2.4; **Items 6–11** from Subheading 2.5; **Item 5** from Subheading 2.7; **Items 4** and **6** from Subheading 2.11.
2. DSP (Lomant's Reagent, ThermoFisher Scientific).
3. Triton x100.
4. Primary antibody: Anti-Tyrosine Hydroxylase Antibody (EMD Millipore, AB152).
5. Secondary Antibody: Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (ThermoFisher Scientific, A-11034).

3 Methods

3.1 LCM Workflow: Sample Acquisition

Most LCM projects are based on samples collected during studies on animal models of human diseases, or well characterized human samples from biorepositories. The type of samples, human or animal, defines the focus during planning steps. Due to the nature of human sample collection, molecular integrity in such samples is affected by preanalytical variability, and compromised to a different degree [23–26]. While handling human samples, especially frozen, the main focus should be on tissue protection from further degradation. With animal samples the focus should be shifted to the preservation of sample integrity during necropsy because preanalytical variability can be effectively minimized by establishing and following standard conditions for animal care and sample acquisition. The protocols described below were validated in numerous LCM studies.

3.2 Rodent Sample Collection

1. Design a standard protocol for the entire study, including the source, age and sex of animals, time of acclimatization in the facility before start of the study, housing conditions, number of extra animals for a pilot study, necropsy protocol tailored to the collection of projected LCM targets (*see* **Note 5**).
2. Perform necropsies in accordance with the Guide for the Care and Use of Laboratory animals [27] and approved institutional ACUC protocol.
3. Perform euthanasia by approved methods applicable to the goals of the study.
4. Maintain RNase-free conditions for necropsy setup by using materials from Subheading 2.1 and wearing gloves through the entire procedure to control contamination by external RNases (*see* **Note 6**).

5. Control contamination with internal RNases by short dissection time, and giving priority to removal of tissues with unstable RNA, changing RNase-free instruments between removal of different organs (*see* **Note 7**), ensuring clean dissections (*see* **Note 8**), blotting blood off collected tissues, changing gloves soiled with blood to avoid cross contamination (*see* **Note 9**), and ensuring the absence of hair on collected tissues.
6. Stabilize LCM target organs within 6 min after euthanasia [18] by one of the following methods (*see* **Note 10**):
7. Position the organ in the middle of the cryomold cover with OCT, and place the cryomold on the slurry of dry ice and 2-methylbutane. Wait for OCT media to solidify and keep the mold on the slurry for 10 min prior to transfer onto dry ice.
8. Store OCT blocks in resealable plastic bags at −80 °C prior to sectioning (*see* **Note 11**).
9. Dispense 50 ml of molecular fixative in the RNase-free 100 ml container with the lid.
10. Place dissected tissue into fixative, close the lid, and incubate for 18 h on an orbital shaker at RT (*see* **Note 12**).
11. Trim the tissue if suitable for the study, and place in labeled tissue cassettes.
12. Transfer cassettes in Tissue Stabilizer Concentrate (Qiagen) diluted per manufacturer's instructions, and incubate at +4 °C for 12–24 h.
13. Rinse cassettes in 100% ethanol on a shaker for 1 h and process in the automated processor on the following cycle (Table 1) (*see* **Note 13**).
14. Dispense molten Paraplast into embedding molds, transfer tissue from cassettes to the molds, and embed them.
15. Store FMFP tissue blocks at −20 °C prior to sectioning (*see* **Note 14**).

3.3 Handling and Processing of Human Frozen Repository Samples

1. Make sure that patient information is protected, and all required documents for work with human samples are in place.
2. Handle the tubes with frozen samples on dry ice at all times, with periodic splash of liquid nitrogen on the top of the tubes.
3. Create RNase-free conditions during sample handling.
4. Make sure not to thaw out frozen tissues submitted in 1.8 ml screw cap cryotubes during the division of the sample for different parts of the study. Embed pieces designated for LCM in OCT, as described above (*see* **Note 15**).
5. Embed tissue samples submitted in RNA*later*® in OCT media without division (*see* **Note 16**).

Table 1
Processing protocol for mouse tissues fixed in molecular fixatives

Step	Reagent	Time (min)	Temperature (°C)	Vacuum
1	100% ethanol	60	20	0.5 bar
2	100% ethanol	30	20	0.5 bar
3	100% ethanol	30	20	0.5 bar
4	100% ethanol	30	20	0.5 bar
5	100% ethanol	30	20	0.5 bar
6	Isopropanol	30	20	0.5 bar
7	Isopropanol	30	20	0.5 bar
8	Xylene	30	20	0.5 bar
9	Xylene	30	20	0.5 bar
10	Paraplast	30	56	0.5 bar
11	Paraplast	60	56	0.5 bar

3.4 LCM Workflow: QC of Molecular Integrity in Acquired Tissues and Pathology Evaluation of Tissue Blocks for LCM Objectives

The control of RNA quality of the starting material is mandatory not only because the high-quality samples guarantee the validity of downstream results, but also for proper choice of downstream analysis applicable to the degree of RNA degradation in the tissue blocks, especially in case of unique and frozen samples, as well FMFP tissues. Not checking sample quality and processing an unsuitable sample through the entire LCM workflow is costly and time consuming. Samples of low RNA quality should be excluded from the sample set or analyzed with the samples of similar quality. We do not routinely check DNA and protein quality in the samples collected under conditions established for RNA preservation (*see* **Note 6**). Molecular quality of archival FFPE blocks is variable. Moreover, the molecular integrity in LCM targets in FFPE tissues depends on target cell type composition and location of this target in the block. Thus, we check quality of RNA and DNA in LCM FFPE targets after purification of molecules, and then select samples suitable for prospective downstream molecular analysis (*see* **Note 17**). Phosphorylated proteins are not preserved in FFPE tissues, however, TheraLin and PAXgene can be used instead, for both human and rodent tissues [28, 29]. We combine, in the same step of the LCM workflow, tissue sectioning for QC of tissue blocks and production of hematoxylin and eosin (**H&E**) stained slides for initial pathology evaluation. The data about presence, number, and size of the desired targets in tissue blocks should be used to plan the next steps of the LCM workflow.

1. Maintain RNase-free conditions throughout the procedure as previously described [18], including (a) RNase AWAY™ to wipe all the surfaces and tools except the cryostat chamber, (b) 100% ethanol to wipe the cryostat chamber, (c) a new blade for each block, (d) trimming and discarding 20 μm of tissue from the face of the previously cut block at the start of the sectioning (*see* **Note 18**), (e) sectioning one OCT block at a time after 30 min equilibration to the cryostat temperature (f) sectioning one FMFP block at a time after 5 min equilibration to RT (g) using 1.5 ml nuclease-free micro-centrifuge tubes for sections.
2. For *paraffin embedded tissues* use a microtome at RT. Prepare three labeled tubes, cut three 10 μm paraffin sections for each tube, and place tubes with sections on dry ice. Spread the next section on a water bath, mount on a glass slide, and dry at RT overnight for H&E staining.
3. For *OCT tissue blocks*, set the cryostat chamber and object holder temperature at −17 °C and −16 °C, respectively (*see* **Note 19**).
4. Place three labeled 1.5 ml nuclease-free micro-centrifuge tubes and RNase-free forceps in a small container with dry ice inside the cryostat for the collection of three replicates of frozen sections.
5. Face the block, cut three 10 μm sections, and put them in a tube kept in dry ice container using RNase-free forceps. Move the tube into the box with dry ice and repeat the procedure for other replicates (*see* **Note 20**).
6. Cut and mount the next section on a glass slide for future pathology evaluation, and move it onto dry ice (*see* **Note 21**).
7. Wrap the block in aluminum foil, and place on dry ice. At the completion of sectioning, place the blocks in resealable bags and transfer them from the box of dry ice to −80 °C storage.
8. Prepare all the required components of a selected RNA extraction kit, including DNase treatment reagents.
9. Working with each tube one at a time, warm up the cap with your fingers keeping the tube in dry ice. Open the cap, add 350 μl of lysis buffer to the tube with ***OCT sections***, vortex the tube for 2 min at maximum setting, place in wet ice, and repeat the procedure with two remaining tubes. To the tube with ***FMFP sections*** add 1 ml of xylene, vortex the tube for 2 min at maximum setting, centrifuge at 8000 × *g* for 1 min, aspirate xylene, and repeat the procedure. Add 1 ml of 100% ethanol, vortex the tube for 2 min at maximum setting, centrifuge at 8000 × *g* for 1 min, aspirate ethanol, and repeat the procedure. Dry the pellet for 5 min in a fume hood.

10. For ***FMFP tissues***, continue RNA extraction with PAXgene Kit following the protocol in Subheading 3.26, steps 1–17 and using a new collection tube after each protocol step (*see* **Note 22**).
11. For ***OCT tissue*** lysates use RNeasy Micro Kit, following the protocol from Subheading 3.25, steps 1–16.
12. Determine RNA concentration in the samples using Nanodrop with 1.5 μl sample input.
13. Dilute the samples with RNase-free water to the concentration below 5000 pg/μl and load them on Pico Chip (*see* **Note 23**). Determine RNA Integrity Number (**RIN**) in RNA samples based on Agilent Bioanalyzer run [30, 31].
14. Exclude the samples with low RNA integrity from the sample set.
15. Stain the prepared slides with H&E, coverslip and air-dry at RT overnight (*see* **Note 24**).
16. Scan the slides into the Aperio database, place them in a designated study folder, and annotate the LCM targets using the ImageScope software, per the user's guide.
17. Based on the size and morphology of annotated targets designate the samples for the whole section manual cutout/scrape, IR or UV laser LCM (*see* **Note 25**) (Fig. 1a–c).
18. Assess the need to trim the block based on the pathology annotation (*see* **Note 26**).
19. For Pilot Study, select the blocks with the largest annotated area for each LCM target (*see* **Note 27**).

3.5 LCM Workflow: Pilot Study

A pilot study is an integral part of LCM workflow which provides all the necessary information for further decisions about the main study and its successful completion. The following issues should be addressed in the pilot study: (a) suitable necropsy (animal studies) or sample handling/processing (human tissues) protocol, (b) RNA/DNA/Protein quantity per unit of LCM target area (c) estimating the number of sections/slides needed to satisfy the yield requirements of downstream analysis, (d) LCM slide preparation protocol, (e) LCM dissection approach and protocol, (f) lysis and extraction protocol, (g) sample suitability for prospective downstream analysis. Prior to the main study, the purified molecules from the pilot must be analyzed by the chosen downstream platform, and any problems encountered in each step of LCM workflow should be troubleshooted, accordingly.

1. Based on annotation data in Aperio ImageScope software, information about RNA content in the target [18] (*see* **Note 28**) and downstream input requirements, estimate a number of sections needed for dissection per sample.

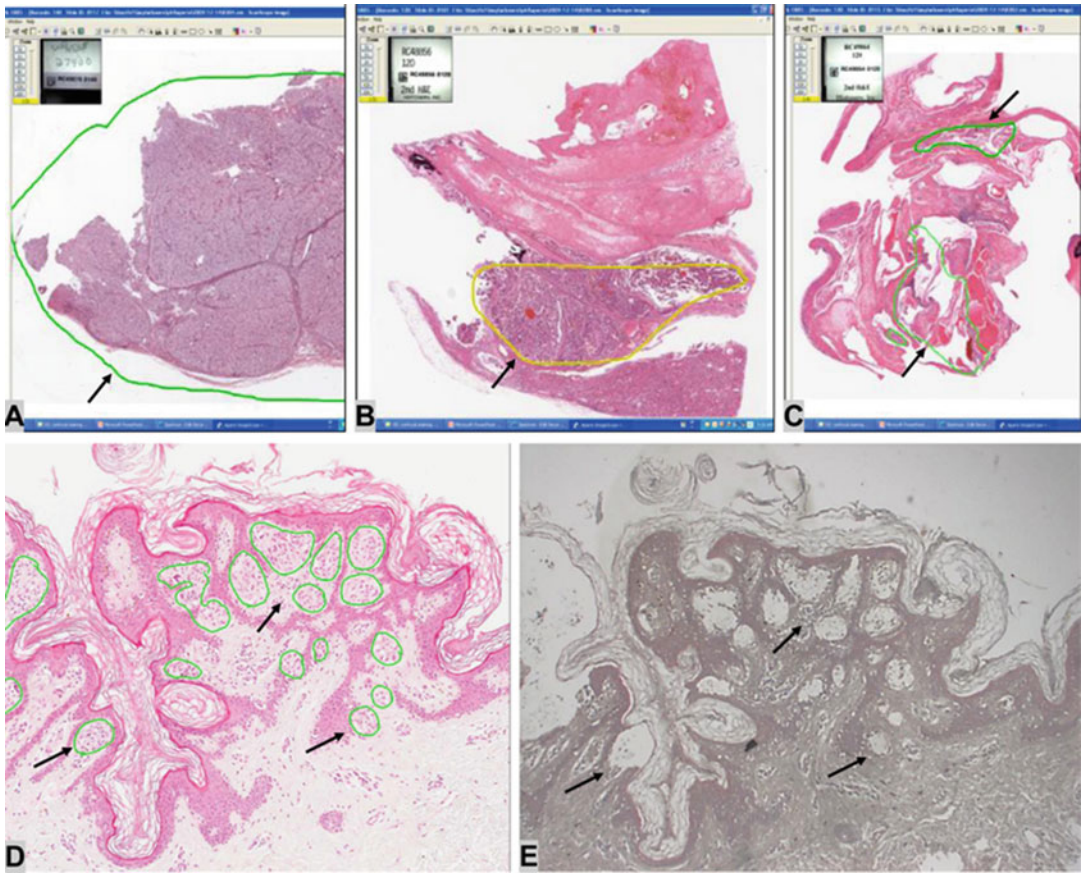

Fig. 1 Role of annotated digital images and LCM reference slide in LCM workflow. (**a**) The whole annotated (arrow) section is an LCM target of interest. A stained slide can be hand macrodissected (cut with a laser blade out of a membrane, or scraped with a blade off a glass slide). (**b**) The annotated LCM target (arrow) is large but requires separation from non-targeted adjacent tissue. The best approach is laser cutting on a membrane slide. (**c**) Multiple target areas of different size and morphology are present (arrows). Depending on size and significance of small targets, the slide could be subjected to both techniques (UV laser cutting and IR laser capture) on a glass membrane slide. (**d**, **e**) Navigated dissection of human skin biopsy: LCM reference slide (**d**) of human skin biopsy with annotated targets (melanoma, arrows) and a subsequent unstained LCM slide with dissected targets (arrows) after reflection of reference annotations onto a live image of LCM section (4× magnification, Arcturus XT)

2. Cut required number of sections from each of the blocks selected for the LCM pilot (*see* Subheading 3.4, **step 19**) at thickness suitable for sample and tissue type and mount sections on a type of slide appropriate for the dissection of the target (*see* Subheading 3.8, **steps 1–9**, Subheading 3.9, **steps 1–5**, Subheading 3.10, **steps 1–6**). Mount the last section on glass slide for H&E reference (*see* **Note 29**).
3. Stain the slides with CVAE stain suitable for the tissue type (*see* Subheading 3.12, **steps 1–7**, Subheading 3.13, **steps 1–7** and Subheading 3.14, **steps 1–4**).

4. Dissect the selected slides within a 20 min time frame and determine the number of slides for simultaneous loading in stage slide holder (*see* **Note 26** *and* **Note 30**).
5. Prepare lysates by acceptable method from Subheading 3.19, steps **1–6**, Subheading 3.20, **steps 1–3**, Subheading 3.21, steps 1–8, Subheading 3.22, **steps 1–8** and Subheading 3.23, steps **1–2**.
6. Extract the molecules of interest by the acceptable method from Subheading 3.25, steps **1–16**, Subheading 3.26, **steps 1**–17, Subheading 3.27, **steps 1**–7, Subheading 3.28, **steps 1–9** and Subheading 3.29, steps **1–9**.
7. Calculate yield in ng/mm² for the target type as an average of two replicates, and add 30% of this number to the average yield, to account for loss of RNA/DNA during extraction.
8. Conservatively estimate the number of slides needed for LCM of each target, based on the cumulative area of targets on the annotated reference H&Es, and the average yield from **step 7**.
9. If the molecular content of LCM sample satisfies the input requirements of the downstream application, run the analysis to confirm the suitability of the sample for the selected downstream platform; otherwise troubleshoot LCM workflow and acquire the suitable LCM sample.
10. If further LCM workflow evaluation is required, prepare more slides from the same block.
11. Record the optimized conditions for use in the main study.

3.6 LCM Workflow: Preparation of LCM Slides for Main Study

Preparation of LCM slides for dissection includes two steps: (a) sectioning of tissue blocks and mounting the sections on LCM slides, (b) staining of LCM slides for the visualization of targets on the LCM dissecting screen. RNase-free conditions should be maintained for both the steps. The choice of LCM slides depends on method of tissue preparation, target size, type of LCM instrument, type of tissue and LCM targets, as well as difficulty of microtomy/cryotomy (*see* **Note 31**).

The choice of an RNA-friendly LCM stain should be based on visualization of the LCM target on the dissecting screen. Although there are different stains available [19, 20, 32, 33], we found that one of these three stains: Modified H&E, MethylGreen, or Cresyl violet/EosinY mix (**CVAE**) [15, 18, 21, 34–36] (*see* **Note 32**), will satisfy dissecting screen visualization requirements for any LCM project.

The described staining protocols are RNA-friendly and applicable to wide range of human and rodent tissue types. In this section, we also address LCM slides preparation from FMFP blocks, considering the high molecular quality of such samples in combination with acceptable morphology for pathology evaluation and good visualization on LCM dissecting screen (Fig. 2a–d, Table 2).

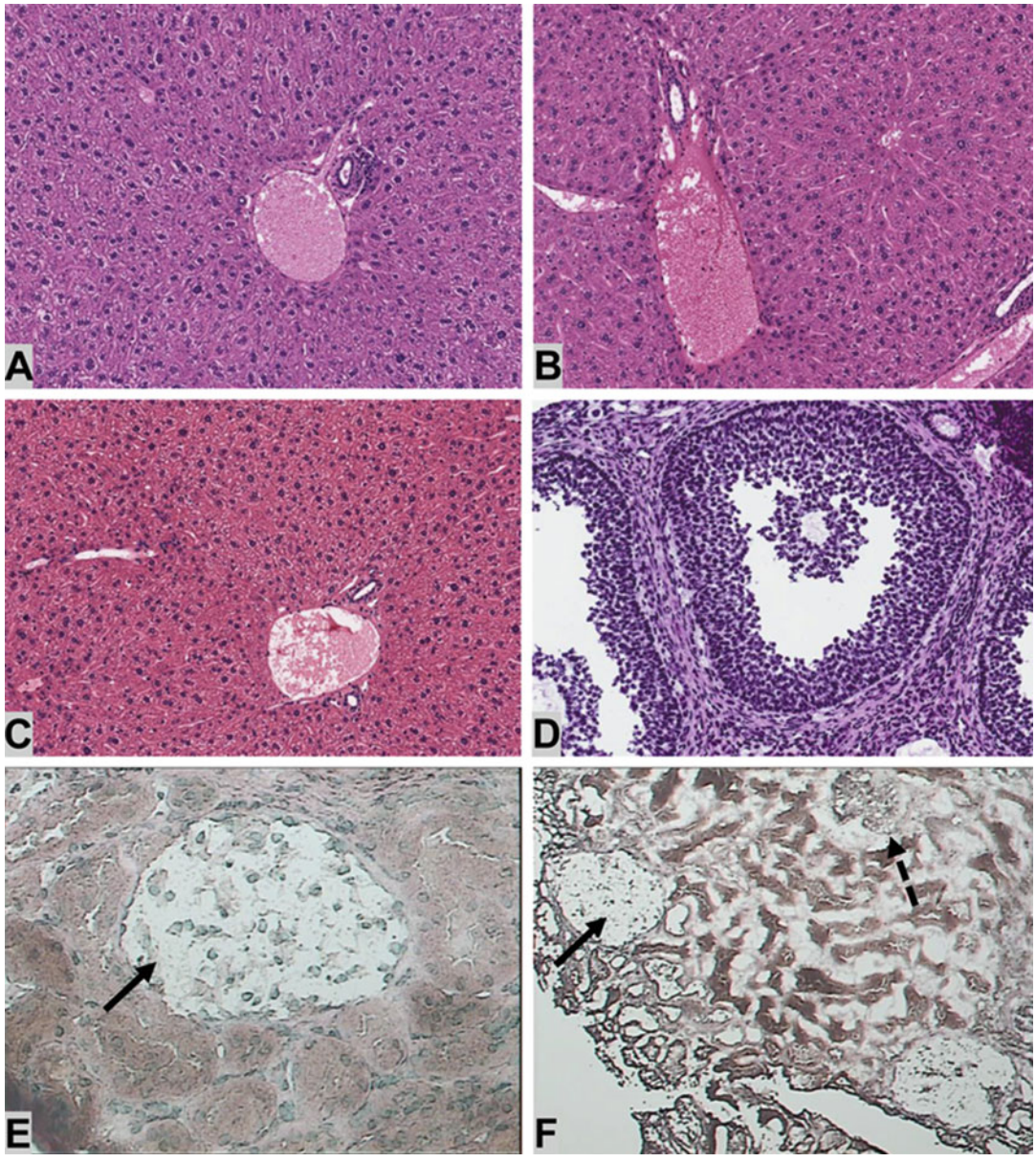

Fig. 2 Morphology of FMFP and RNA*later* preserved tissues. (**a**–**c**) H&E of mouse liver fixed in PAXgene (**a**), STUMol (**b**) and DSP (**c**) (Aperio image, 10×). (**d**) H&E of OCT section of mouse ovary fixed in DSP (Aperio image, 10×). (**e**, **f**) PixCell IIe LCM screen view of CVAE stained OCT section of human kidney: (**e**) fresh frozen section after dissection and removal of a glomerulus (arrow, 10× objective), and RNA*later* preserved (f) tissue section with a glomerulus before (dashed arrow) and after dissection and target pick up (solid arrow) (4× objective)

Table 2
RNA integrity in selected FMFP mouse tissues

Tissue type	Fixative	RIN
Liver	DSP	8
Liver	TheraLin	5.1
Tumor/pancreatic	TheraLin	5.2
Liver	STUMol	9.7
Tumor/pancreatic	STUMol	9.8
Liver	PAXgene	7.5
Tumor/pancreatic	PAXgene	7.5
Ovary	PAXgene	6.7
Stomach	PAXgene	9.7
Intestine	PAXgene	9.6
Lung	PAXgene	9.8
Skin	PAXgene	7.6
Tongue	PAXgene	9.2

3.7 Sectioning of OCT, FMFP, and FFPE Tissues

The RNase-free conditions described in **step 1** of Subheading 3.4 are applicable to both microtomy and cryotomy. Other conditions unique for frozen or paraffin-embedded tissues are described in the respective protocols below. We mount frozen sections intended for IR laser capture on glass CryoJane slides, and paraffin sections for IR dissection, on glass membrane slides. For UV laser cutting, we use paraffin sections on metal frame membrane slides, however, frozen sections we mount either on frame membrane slides or manually prepared CryoJane membrane slides. Despite the type of slide and tissue block, we cut the required number of sections per sample (calculations are based on data from pilot study plus two extra slides) consecutively as serial sections, mounting every sixth slide on glass slide for LCM reference (*see* **Note 33**).

Microtomes and cryostats with automated functions, as well as anti-roll plate for cryosectioning, are preferred for LCM slide preparation because they create sections of uniform thickness, which benefits staining appearance and laser focusing. The quality of LCM sections is crucial for successful microdissection. Microtomy and cryotomy is a skill which requires substantial training; seek help from professional histology services if sectioning expertise is lacking.

3.8 OCT Blocks: Fresh Frozen or DSP-Fixed Tissues on CryoJane Slides

1. Precool CryoJane Adhesive Coated Slides, AdhesiveTapeWindows and a Hand Roller in the cryostat chamber for 30 min [21], keep them inside the cryostat for the duration of sectioning, and follow the procedure below.
2. Set the object holder and cryostat chamber temperature to −24 °C and −29 °C, respectively, and section thickness to 8–10 μm (*see* **Note 34**).
3. Equilibrate OCT block to cryostat temperature for 30 min (*see* **Note 35**).
4. Take a slide out of the wrapper, label it with the pencil, and place on the mounting pad of UV Flash Unit.
5. Mount the block into the object holder, face it, apply adhesive tape to the face of the block, and firmly roll it onto the block with the hand roller.
6. Cut a section using the automated function of the cryostat, immediately transfer the section bonded to the tape onto the adhesive slide, and roll it onto the slide by two or three passes of a hand roller.
7. Transfer the slide to the UV pad of UV Flash Unit, and flash it with UV light.
8. Carefully peel the tape off the slide starting from one of the corners, and transfer the slide in the slide box on dry ice (*see* **Note 36**).
9. Transfer the packed slides and blocks to −80 °C storage out of the box with dry ice.

3.9 OCT Blocks: Fresh Frozen or DSP-Fixed Tissues on Membrane Slides

1. Label the slides with solvent resistant adhesive labels on the "window" side, incubate in a UV chamber together with SupportSlide at 352 nm for 30 min (*see* **Note 37**), and follow the procedure below.
2. Insert the chuck with OCT block in an object holder and face the block.
3. Place the membrane slide in the cryostat chamber for 2 min, and cut the section with cryostat automated function using the anti-roll plate. Insert SupportSlide into the "window" of membrane slide and mount the section onto the membrane side of SupportSlide-membrane slide assembly (Fig. 3a–d). Remove the membrane slide with mounted section from the slide assembly and put on dry ice keeping the lid on the box at all times (Fig. 3e). Maintain RNase-free conditions for the SupportSlide for continuous use with subsequent sections.
4. Place the slides in Five-Slide Mailers on dry ice (Fig. 3e), detach the block and wrap it in aluminum foil, and place slide boxes and blocks in resealable bags on dry ice.
5. Transfer slides and blocks to −80 °C storage.

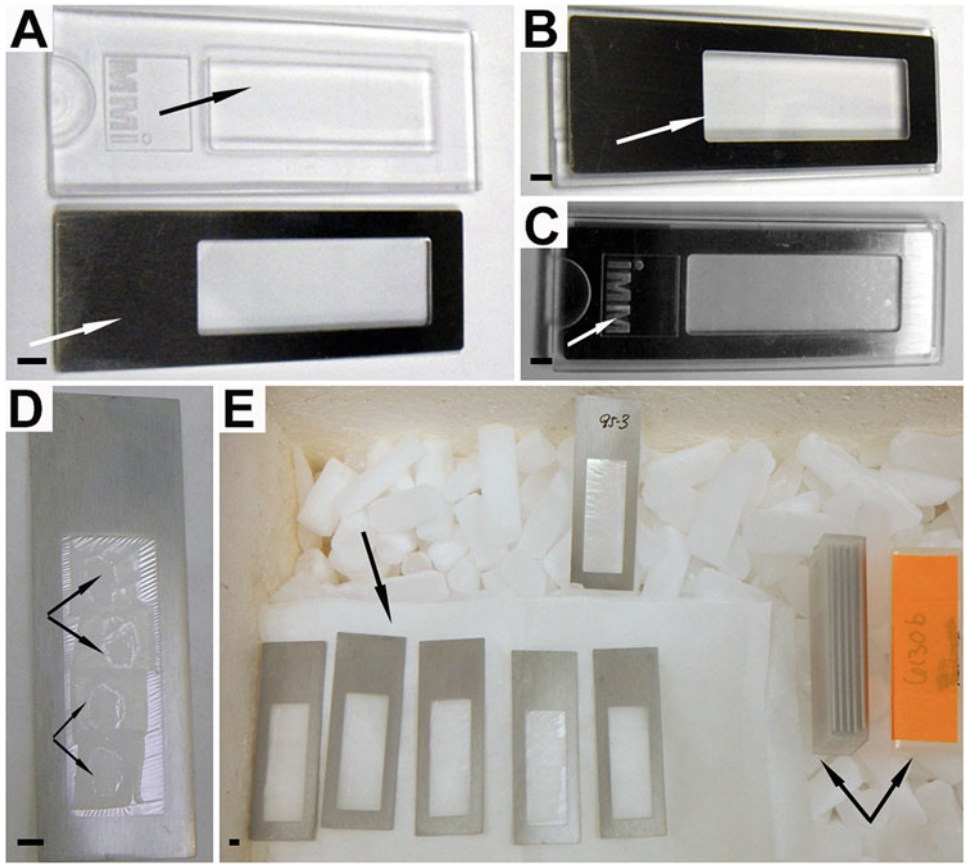

Fig. 3 LCM slide preparation: Mounting of OCT tissue sections on frame membrane slides. (**a**) MMI SupportSlide with the elevated platform (black arrow) facilitates section mounting on frame membrane slide (white arrow). (**b**) The window of frame membrane slide (arrow) snugly fits the elevated platform of SupportSlide. (**c**) SupportSlide-frame membrane slide assembly should be flipped that inverted MMI logo of SupportSlide (arrow) faces cryotomist during mounting of the section. (**d**) The OCT block trimmed close to the tissue allows us to fit several serial sections (arrows) in the window of a frame membrane slide. (**e**) Immediately after mounting, pre-labeled slides should be placed on a Kimwipe in a Styrofoam box with dry ice, label down (solid arrow). Slides should be transferred in prechilled Five-Slide Mailers (double arrows) for −80 °C storage prior to LCM. Scale bars correspond to 4000 μm. (reproduced from Open Access ref. 15)

3.10 Paraffin Blocks: FMFP and FFPE Tissues on Membrane Slides

1. Label glass or frame membrane slides and charged++glass slides for reference H&Es (*see* **Note 38**). Expose the slides to 352 nm UV light for 30 min
2. Set a water bath to 43–44 °C. Cover the bottom of a new weigh boat with a folded Kimwipe, pour in 10 ml of RNase-free water, and place into ice pan. Set microtome section thickness to 7 μm.
3. Trim the block according to the trim template, insert into the block clamp, face and put it cut side down into the boat. Soak the block for the length of time established in the pilot study (*see* **Note 39**) (Fig. 4a, b).

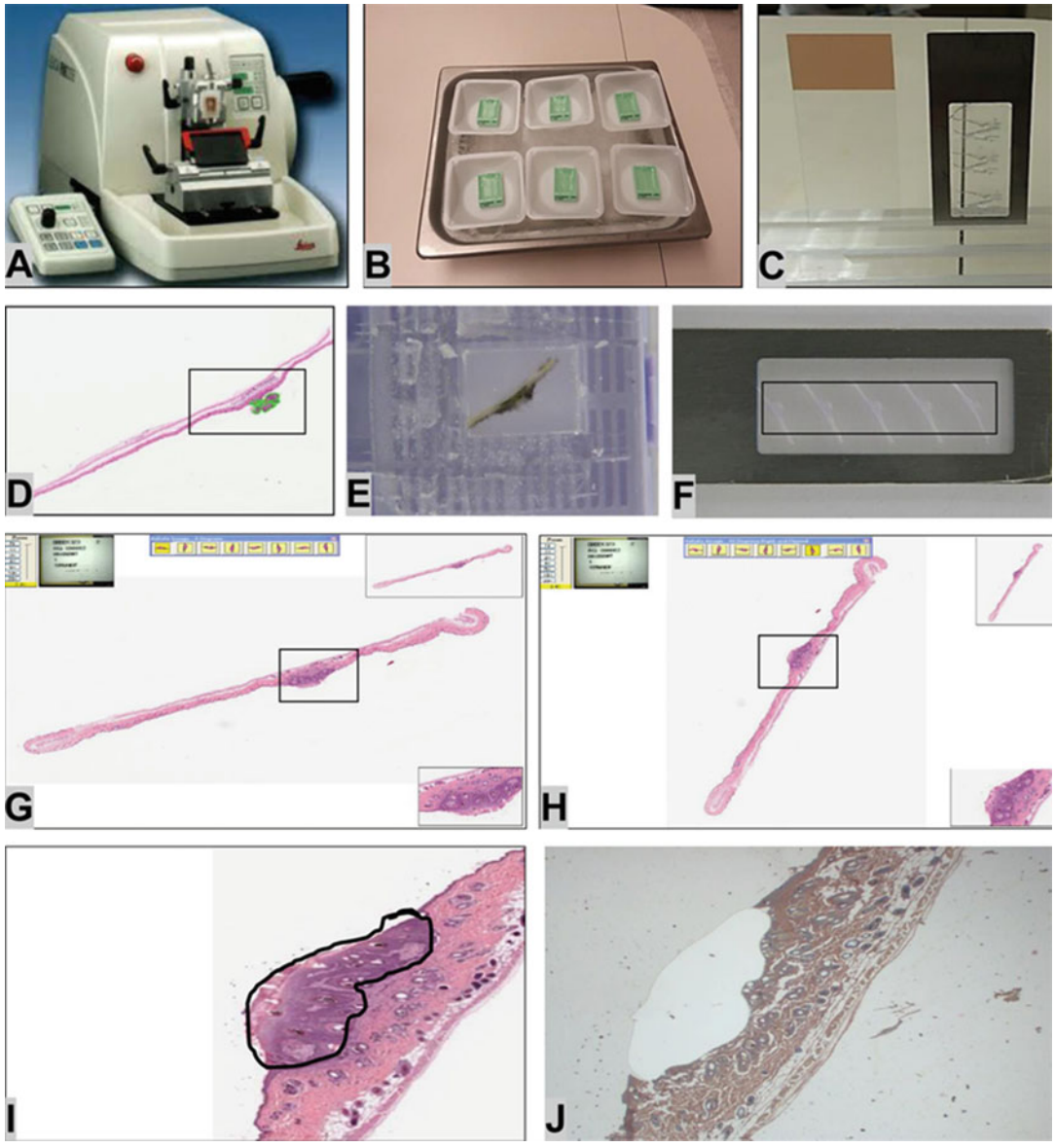

Fig. 4 Paraffin block microtomy and incorporation of ImageScope™ digital slide view in LCM workflow. (**a**) Automated microtome with mounted paraffin block. (**b**) Soaking of paraffin blocks on ice. (**c**) Drying glass and membrane slides after section mounting. (**d**) Paraffin block trimming template. (**e**) View of the paraffin block trimmed per the template. (**f**) Serial sections fitted on a frame membrane slide after block trimming. (**g**) Digital image of annotated reference H&E section in ImageScope™ Main Window. (**h**) Digital image rotated to match the orientation of LCM section on dissecting screen during laser cutting. (**i**) Annotated LCM target on reference H&E image enlarged to match the target magnification (4×) on LCM dissecting screen. (**j**) The view of LCM section on MMI CellCut screen (4×) after reflection of reference annotation to the live image followed by laser cutting. Target area in (**d**), (**g**), and (**h**) is indicated by a *rectangle* (Aperio screen 0.4× magnification)

4. Cut a paraffin ribbon with automated function of the microtome, spread it on the water bath, and mount sections as soon as the LCM target area is flat, without wrinkles.
5. Position the tissue sections on the flat side of the frame membrane slide so that the LCM target area is located in the frame window along the midline (Fig. 4f).
6. Dry the sections in an upright position for 30 min (Fig. 4g), and absorb water accumulated in the bottom of the slide window with the filter paper. For *FFPE tissues*, continue drying overnight at RT, covered with a Kimwipe to avoid contamination. Store FFPE slides at +4 °C or in a desiccator prior to microdissection. For *FMFP tissues*, continue drying in a desiccator for 1 h, and store them in Five-Slide Mailers at −20 °C prior to microdissection.

3.11 LCM Workflow: Staining of LCM Sections Mounted on Membrane and CryoJane Slides

Staining should be performed in a fume hood. RNase-free conditions should be maintained during the staining procedure: (a) RNase-free reagents, (b) RNase-free water for the preparation of solutions, (c) RNase-free forceps and staining blocks, (d) RNase and protease inhibitors for water containing solutions, (e) fresh solutions after each batch of six slides. Staining for both frozen and paraffin sections is performed in 50 ml Falcon tubes filled with 45 ml of reagents using the same protocol steps after stain application to the slide (Fig. 5a–i). Below, we describe our one step CVAE [15, 18, 21, 34, 37] LCM staining protocol for frozen and paraffin sections on membrane and CryoJane slides. Modifications of the staining protocol for LCM H&E are described in **Note 24**.

3.12 Paraffin-Embedded Tissue Sections on Frame and Glass Membrane Slides

1. Prepare vortexed CVAE stain, containing 75 µl of CVA, 25 µl of eosin Y, 250 µl of RNase-free water, and 250 µl of 100% ethanol. Keep the stain at RT, and pipette from the surface, avoiding the precipitate on the bottom of the tube.
2. Move the box with the slides from −20 °C freezer to +4 °C refrigerator for 20 min, then open resealable bag and transfer the box to wet ice.
3. Move the slide (*see* **Note 40**) in a desiccator for 15 min before staining.
4. Incubate the slide in two changes of xylene for 5 min each, then in two changes of 100% ethanol for 1 min each (Fig. 5a, b).
5. Dispense 300 µl of vortexed CVAE containing 1 µl of ProtectRNA onto the section using barrier pipette tip, incubate for 30 s (*see* **Note 41**), and drain the slide on a Kimwipe (Fig. 5f).
6. Incubate the slide in two changes of 100% ethanol for 30 s each, then in two changes of xylene for 3 min each, and air-dry for 5 min in a vertical position protected from contamination (Fig. 5g, h).

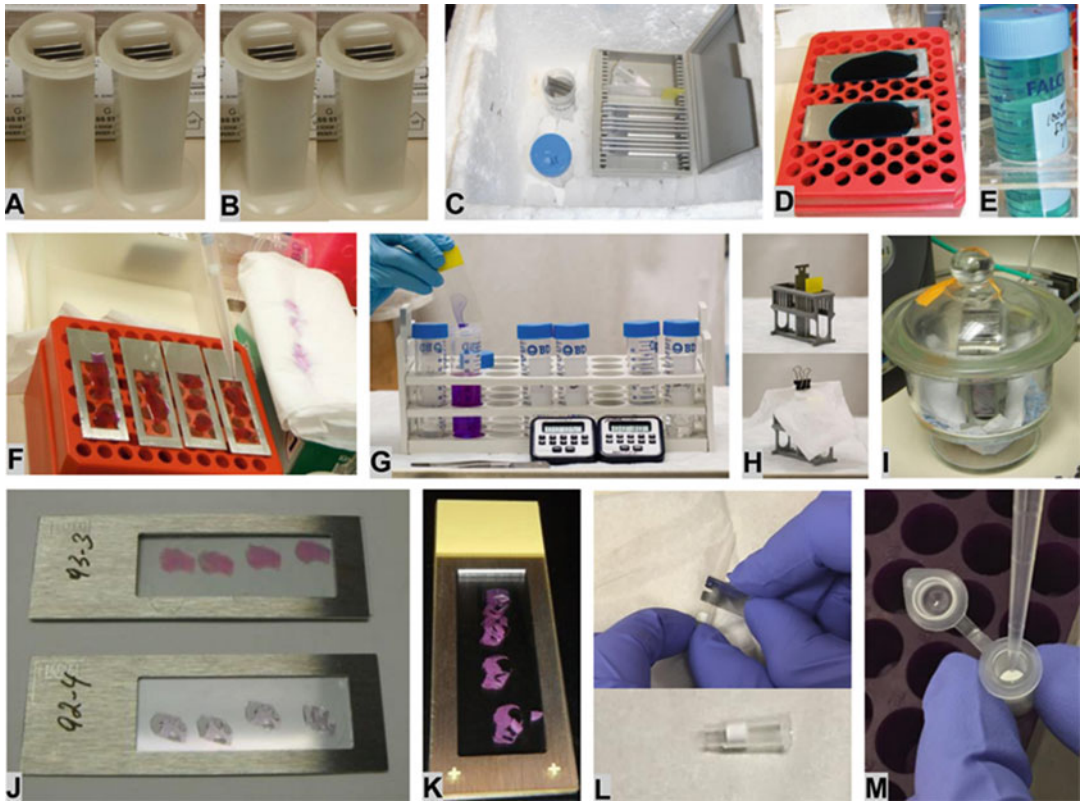

Fig. 5 LCM slide staining, cutouts handling after LCM and removal from LCM lysate. (**a**, **b**) Deparaffinization of paraffin sections in two changes of xylene (**a**) followed by incubation in two changes of 100% ethanol (**b**) prior to stain application. (**c**–**e**) Fresh frozen OCT tissue section fixation in LCM fixative (**c**) followed by OCT removal with MethylGreen (**d**) and subsequent rinse with 100% ethanol (**e**) prior to stain application. (**f**) Stain application onto frame membrane slides. (**g**) Reagent column for slides rinse after staining and subsequent dehydration with 100% ethanol and xylene. (**h**, **i**) LCM slide air drying followed by incubation in a desiccator prior to LCM, respectively. (**j**) Stained frame membrane slides with serial sections. (**k**) Assembly of LCM dissected slide and RNase-free glass slide for LCM slide transfer from LCM stage to the conventional dissecting microscope. (**l**) Making a filter from the barrier pipette tip. (**m**) Transfer of LCM lysate to the filter-centrifuge tube assembly

7. Transfer the slide into a desiccator at least for 15 min prior to microdissection (Fig. 5i) (*see* **Note 42**).

3.13 Frozen OCT Sections on Frame and Glass Membrane Slides

1. Prepare a fixative solution of 3% glacial acetic acid in 100% ethanol and place in Styrofoam box with dry ice for 1 h.
2. Prepare vortexed CVAE stain, combining 75 μl of 1% alcoholic CVA, 25 μl of eosin Y, 450 μl of RNase-free water, and 450 μl of 100% ethanol. Keep the stain at RT, and pipette from the surface, avoiding the precipitate on the bottom of the tube.

3. Inside the Styrofoam box, transfer LCM slide from Five-Slide Mailer or slide box into the tube with fixative, and incubate for 30 s (Fig. 5c).
4. Apply 1 ml of OCT removal solution (vortexed mixture of 996 μl of MethylGreen and 4 μl of ProtectRNA) to the slide using barrier pipette tip and incubate for 20 s. Drain the slide and repeat the procedure (Fig. 5d).
5. Rinse the slide in 100% ethanol for 10 s with up and down movement (Fig. 5e).
6. Apply 300 μl of CVAE to the slide using barrier pipette tip and incubate for 30 s, drain on Kimwipe (Fig. 5f). Skip this step when using MethylGreen as LCM stain.
7. Incubate the slide in two changes of 100% ethanol for 30 s each, then in two changes of xylene for 3 min each (Fig. 5g).
8. Dry the slide in a fume hood for 5 min and transfer it in a desiccator for at least 15 min prior to laser dissection (Fig. 5h, i).

3.14 Frozen OCT Sections on CryoJane Slides

1. Place the capped tube with acetone in dry ice for 1 h inside the Styrofoam box. Prepare a fixative solution of 3% glacial acetic acid in 100% ethanol, and place the tube with fixative in wet ice inside the Styrofoam box.
2. Inside the Styrofoam box with dry ice, transfer the LCM slide from Five-Slide Mailer into the tube with cold acetone, and incubate for 15 s.
3. Transfer the slide into the tube with fixative and incubate for 1 min.
4. Follow **steps 4** from Subheading 3.13.

3.15 LCM Workflow: Dissection of LCM Slides

The success of laser microdissection greatly depends on a combination of several conditions: (a) quality of LCM slide, (b) knowledge of software, (c) speed of dissection, (d) precision of target separation from surrounding tissue, (e) presence of nonspecific tissue on the collection cap after target pick-up, (f) the nature and shape of LCM target, (g) adherence of tissue section to the slide, (h) dissection approach. There is a set of microdissection steps common for all LCM instruments on the market: setting up slide borders, creating slide overview, locating and annotating the cells of interest, setting up laser power, focus, speed and the width of laser cutting line, loading the collection device, and locating and activating the laser. However, the collection device is specific to the LCM instrument. It is often challenging to accumulate large numbers of cutouts for a single sample for molecular extraction with particular type of device, or cutouts are too large for the collection device. To overcome these challenges, we use different dissection approaches

and a conventional dissecting microscope for the removal of nonspecific contamination from the cap and transfer of cutouts into the tube with lysis buffer. Prior to LCM session it is advisable to create a list of all the required materials and organize them for the uninterrupted LCM procedure. Below we describe a dissection protocol for IR laser capture and UV laser cutting.

3.16 IR Laser Capture Microdissection

We perform IR LCM of OCT tissues on CryoJane slides and paraffin-embedded tissues on glass membrane slides, which provide for complete pick-up of laser-captured targets (*see* **Note 43**).

1. Open a pathology annotated image on a reference computer screen.
2. Load LCM cap cartridge with caps of choice into the stage cartridge slot (*see* **Note 44**), create a folder for project documentation, and set up a timer for the duration of dissection established in a pilot study (*see* **Note 45**).
3. Load a test slide in the stage slide holder, bring tissue into focus with 2×-objective, and use ImageScope software to rotate the annotated H&E image on the Reference Computer monitor to match the view of the section on LCM dissecting screen (Fig. 4g, h, j). Optimize image quality on LCM screen per manufacturer's instructions, and remove the slide.
4. Take the LCM slide out of the desiccator, load in the slide holder, and start the timer.
5. Acquire slide overview, position the cap on the target area, and change to the 10×-objective. Match the magnification of the LCM reference image and LCM screen image (Fig. 4i, j).
6. Locate the laser and adjust the size of the laser melting spot per the manufacturer's user guide (*see* **Note 46**).
7. Reflect the target annotations from Aperio reference image onto the image on the dissecting screen, and save the image in the project folder.
8. Activate the IR laser to dissect marked targets.
9. Move the cap to the QC station and inspect for pickup efficiency and nonspecific contamination. Save the image of dissected targets and the section after dissection.
10. Transfer the cap under a conventional dissecting microscope, and remove any nonspecific contamination from the cap. Cut the film around the captured targets using an RNase-free blade (Fig. 6g–i), and continue with the lysis procedure.
11. If several slides were stained simultaneously for LCM session, repeat **steps 4–10** for the remainder of the slides.

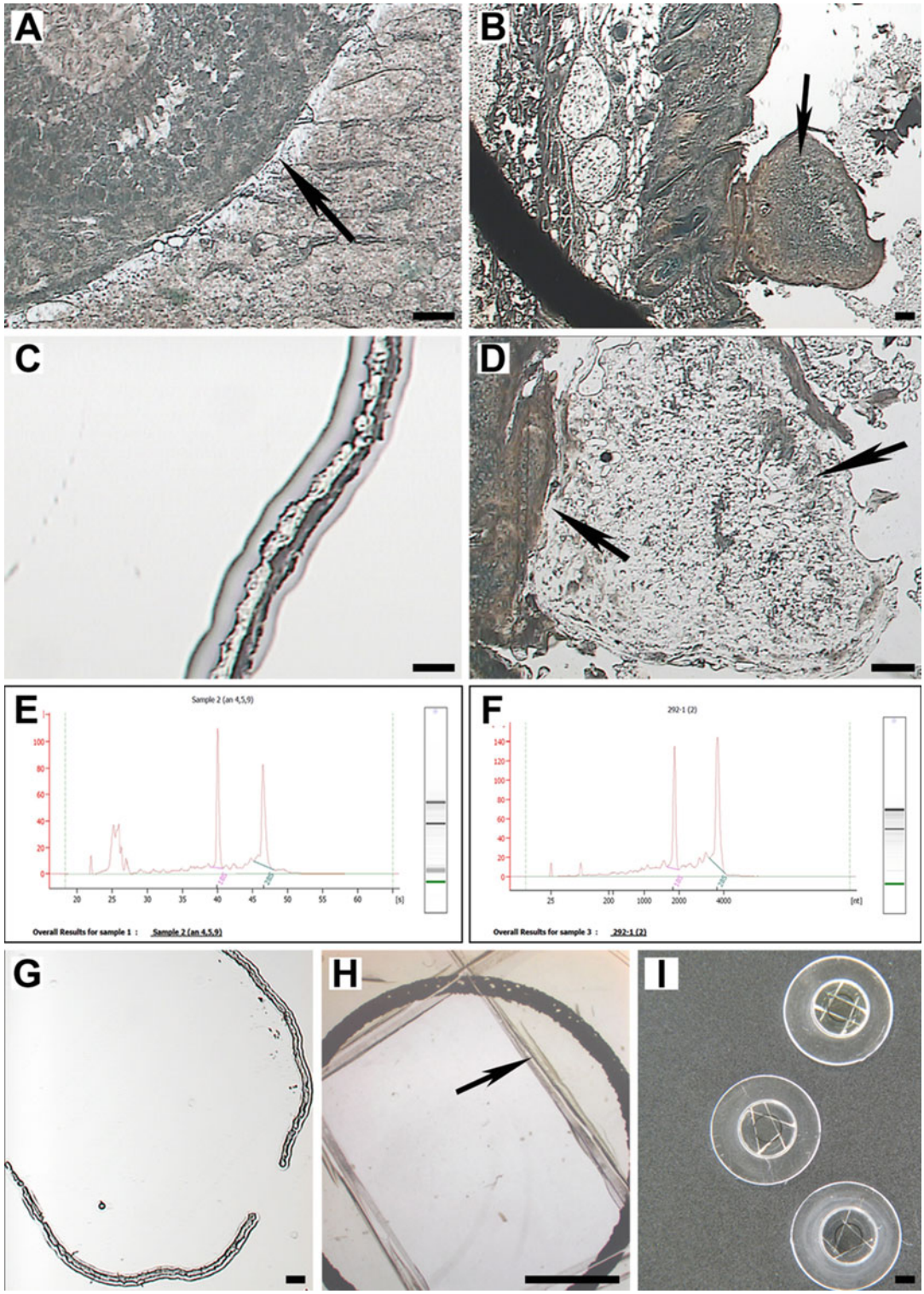

Fig. 6 IR LCM on glass CryoJane slides (Methyl Green stain) and quality of RNA retrieved from LCM targets. (**a**) IR LCM of mouse normal ovarian epithelium: arrow indicates the target area after removal of captured

3.17 UV Laser Cutting

We perform UV laser cutting on metal frame membrane slides with MMI CellCut (*see* **Note 47**).

The protocol for the Arcturus XT is the same, with the exception for laser location and focusing pertaining to the instrument. We dissect glass membrane slides on Arcturus XT only for cases requiring capture and cutting of targets from the same section. Nonspecific contamination during UV laser cutting on frame membrane slides is extremely rare, and happens only due to poor adherence of fat or collagen to the membrane. This issue can be addressed during LCM slide preparation, or by altering the dissection approach (*see* **Note 48**).

1. Open a pathology annotated image on a reference computer screen.
2. Create a folder for project documentation, and set up a timer for the duration established in the pilot study.
3. Load an RNase-free glass slide (*see* **Note 49**) in the stage slide holder frosted side down.
4. Load a test membrane slide on the top of the glass slide, with slide label facing up, making sure that it is leveled in the slide holder (*see* **Note 50**).
5. Define the slide borders, acquire a slide overview, rotate the annotated H&E image on the Reference Computer monitor to match the position of the section on LCM screen.
6. Change to the 10×-objective, set up the illumination through camera settings, bring the membrane without the section into screen view, and focus the laser per manufacturer's instructions.
7. Move LCM target into screen view, bring tissue into focus, draw a line around the target, set laser position, and activate the laser. Adjust laser speed and power to achieve a complete cut and remove the test slide from the slide holder. Save laser settings for the dissection of subsequent slides.
8. Take the first slide out of the desiccator, load in the slide holder, and start the timer.

Fig. 6 (continued) tissue (PixCell IIe, 10×). (**b**) IR LCM of mouse skin (PixCell IIe, 4×): arrow indicates LCM target-papilloma. (**c**) Captured ovarian epithelium on HS Cap (PixCell IIe, 20×). (**d**) View of adhesive left on a slide after removal of captured papilloma (PixCell IIe, 10×): left arrow indicates the papilloma base and right arrow indicates adhesive. (**e**, **f**) Representative Agilent electropherogram of high quality LCM RNA extracted from mouse ovarian epithelium and skin papilloma, respectively. (**g**, **h**) Captured target on HS Cap (PixCell IIe, 4×), and a view of the film after the target area was cut out with a blade and peeled off the HS Cap, respectively: arrow indicates film remaining on the cap. (**i**) View of HS Caps after removal of the film areas with embedded targets. (**a**), (**b**), (**d**), (**g**): Scale bars correspond to 100 mm; (**c**): Scale bar corresponds to 25 mm; (**h**), (**i**): Scale bars correspond to 1000 mm (reproduced from Open Access ref. 21)

9. Load the MMI Cap into a cap holder (*see* **Note 51**).
10. Create a slide overview with 4×-objective, and change to the 10×-objective.
11. Using the slide overview, navigate to the area of interest, bring tissue in focus, reflect the target annotations from reference image onto the image on LCM screen, set laser position, and activate the laser (*see* **Note 52**).
12. Collect the dissected targets with the MMI Cap or with the RNase-free forceps, and continue with the lysis procedure (*see* **Note 53**).
13. Load a new support slide in the stage slide holder for each subsequent LCM slide in the dissection batch, and repeat **steps 7–13** for the remainder of the slides (*see* **Note 54**).

3.18 LCM Workflow: Lysate Preparation from LCM Targets

The method of lysate generation has a profound effect on quality and yield of nucleic acids and proteins. Lysis buffer composition depends on sample type, the downstream application platform, and extraction protocol for molecules of interest. We perform extraction with commercially available kits, and use the lysis buffer from the respective extraction kit for LCM lysate preparation. A crude lysate is preferred, if acceptable by downstream analysis platform because no loss of target molecules due to purification provides greater precision/sensitivity of analysis, but often requires modifications of microdissection and lysis procedure [15, 16]. LCM cutouts from frozen tissues intended for RNA purification must be lysed immediately after dissection (*see* **Note 55**), while cutouts intended for DNA can be stored at −20 °C at least for 2 weeks prior to lysis. Protein analysis requires the collection of a large number of cells [13, 20, 38], and tubes with cutouts are accumulated at −80 °C until completion of LCM from a large number of serial sections. Below we describe the lysis procedure for LCM samples dissected on different platforms and intended for different downstream applications.

3.19 LCM Targets from OCT Sections/ RNA Lysate

1. Prepare lysis buffer (*see* **Note 56**) and labeled 1.5 microcentrifuge tube for each serial slide in LCM batch (*see* **Note 57**).
2. Check the start lysate volume of the RNA extraction protocol, divide it by the number of tubes from **step 1**, and fill the tubes with the resulted volume of lysis buffer (*see* **Note 58**).
3. Transfer dissected targets into lysis buffer according to the target size, type of collection cap, and lysis buffer volume, as described below:
4. ***Transfer of large targets*** (200–500 μm in diameter)

Targets on Arcturus Macro LCM cap: peel the membrane with dissected targets off the cap, and place it in a tube with lysis buffer. Continue with the lysis procedure.

Targets on 1.5 ml MMI Cap: dispense not <100 μl of lysis buffer into the tube, close the cap and invert the tube. Continue with the lysis procedure.

Targets not fitting on the caps: collect cutouts directly from the slide under the conventional dissecting microscope at lowest magnification, using RNase-free forceps, and put them in lysis buffer to continue with the lysis procedure (*see* **Note 59**)

5. ***Transfer of small targets*** (below 200 μm in diameter): use one of the following methods for "individual" or "accumulated" lysate production (*see* **Note 60**):

   Arcturus LCM Caps: Under the dissecting microscope at 1–1.5× magnification, remove the nonspecific contamination, cut the film around the targets with the blade (*see* **Note 61**) (Fig. 6h), peel film with targets off the cap with RNase-free forceps, and place in lysis buffer. Continue with the lysis procedure.

   0.5 ml MMI Caps: For lysis buffer volume over 50 μl, dispense buffer into the tube, close the lid, invert the tube, and continue with lysis procedure. For volumes down to 15 μl, perform the following procedure under the dissecting microscope: (1) dispense 5 μl of 100% ethanol on the of MMI Cap, (2) dislodge cutouts with a pipette tip, (3) press the tube over the cap and centrifuge at 2000 × *g* for 15 s to move the dislodged cutouts onto the walls and bottom of the tube (*see* **Note 62**), (4) keeping tube in a horizontal position, evaporate ethanol in a desiccator or fume hood for 15 min, (5) add required volume of lysis buffer, and continue with lysis procedure. For "accumulated" samples, dispense 10 μl of 100% onto the cap, keeping the tube with the targets from the first slide in wet ice. Cut the cap with cutouts from the next tube, dislodge the targets re-using the ethanol, insert the cap into the tube with the previous cutouts and centrifuge the assembly as above, repeating the procedure for the remainder of the slides and evaporating ethanol at the completion of the LCM session.

   0.2 ml MMI Caps: Under a dissecting microscope, dispense 2 μl of 100% ethanol in the cap of the PCR tube, bring the cutouts on the MMI Cap in contact with the drop of alcohol, making sure that all cutouts were dislodged. Close the cap, and centrifuge the tube at 2000 × *g* for 15 s. Evaporate the ethanol, and add 5–10 μl of lysis buffer to continue with lysis procedure. For "accumulated" samples follow the procedure, described above, but dislodge cutouts by the contact method (*see* **Note 63**).

6. Vortex the tube for 1 min at high setting, incubate at RT for 10 min, vortex for 1 min, and spin down.

7. Remove films or membranes from lysate (*see* **Note 64**) choosing the applicable method below.
8. ***Arcturus LCM cap films***: Trap the edge of the film between the cap and the tube wall, and briefly spin the tube in the centrifuge. Holding the tube in a nearly horizontal position, carefully open the cap, and discard the dry film using a pipette tip.
9. ***Large membrane cutouts in 100–350 µl volume***: Centrifuge the tube at 16,000 × *g* for 30 s, transfer the bulk of lysate to a new tube using 200 µl pipette. Spin down the tube, and remove the rest of the lysate using 20 µl pipette with long tips. Discard the tube with the films.
10. ***Small pieces of film or membrane cutouts in 5–100 µl volume***: Filter the membranes or pieces of film out through the stump of RNase-free pipette tip with the barrier, inserted into PCR or 0.5 ml centrifuge tube (Fig. 5l, m) (*see* **Note 65**).
11. Place the tube with prepared lysate on dry ice in Cryobox, and transfer the box from dry ice to −80 °C storage prior to extraction (*see* **Note 66**).

3.20 LCM Targets from FMFP Tissues/ RNA Lysate

1. Prepare lysis buffer TM1 from Paxgene kit per manufacturer's instructions (*see* **Note 56** for DSP fixative), and divide lysis buffer among 1.5 microcentrifuge tubes for each serial slide in LCM batch based on 150 µl start volume for extraction (*see* **Note 58**).
2. Transfer dissected targets into lysis buffer and lyse the sample using the applicable approach (*see* Subheading 3.19, **steps 4** and **5**).
3. Follow **steps 6–11** from Subheading 3.19

3.21 LCM Targets from FFPE Tissues/ RNA Lysate

1. Transfer dissected targets into a 1.5 ml centrifuge tube by "accumulated" method depending on target size and store them at −80 °C prior to lysis.
2. For all downstream applications, except NanoString (*see* **Note 67**), prepare 160 µl of lysis buffer per extraction based on buffer PKD from FFPE kit; 142 µl of buffer PKD, 10 µl of PK, 8 µl of RNaseOut.
3. Add 160 µl of lysis buffer to the tube with LCM targets, incubate at 56 °C for 68 h with 5 min of shaking at 300 × *g* every 30 min and addition of 10 µl of PK every 24 h of incubation. Scale the reagents in all the steps of the protocol for a different start volume.
4. Incubate the tube at 90 °C for 1 h without agitation.
5. Add 16 µl DNase Booster Buffer and 10 µl DNase I stock solution to the tube, mix by inverting the tube, and spin down to collect residual liquid from the sides of the tube.

6. Incubate at room temperature for 15 min.
7. Add 320 μl of buffer RBC (from the kit) to the tube, mix by light vortexing, remove membranes/films from lysate, and put the tube on dry ice.
8. Store lysates at −80 °C prior to RNA extraction.

3.22 LCM Targets from Frozen and Paraffin-Embedded Tissues/ DNA Lysate

1. Transfer dissected targets into a 1.5 ml centrifuge tube by "accumulated" method depending on target size and store at −20 °C prior to lysis.
2. Use one the following lysis buffers for the study: (a) Lysis buffer from Arcturus PicoPure DNA extraction kit (per manufacturer's instructions) (*see* **Note 68**), (b) buffer AL from Qiagen QIAamp Kit (per manufacturer's instructions), (c) prepared DNA lysis buffer (175 μl per sample) based on TD-S0 Buffer from Autogen Animal Tissue DNA extraction kit containing 10 μl of PK per 1 ml of buffer.
3. Incubate the tubes at 56 °C for 2 h for frozen and FMFP tissues, and for 68 h for FFPE tissues with shaking at 300 × *g* every 30 min and addition of 10 μl of PK to each sample every 24 h of incubation.
4. Incubate the tube at 90 °C for 2 h without agitation.
5. If RNase digest is required, centrifuge tubes for 30 s at 16,000 × *g*. Add 1 μl of 10 mg/ml RNase A to each lysate, vortex, and incubate for 30 min at 37 °C.
6. Centrifuge all the samples at 14,000 × *g* for 3 min at 4 °C.
7. Check that volume remains at 175 μl and bring up the volume difference with nuclease-free water if needed.
8. Proceed with extraction or store samples at −20 °C.

3.23 LCM Targets from Frozen and Paraffin-Embedded Tissues/ Protein Lysate

1. Place LCM caps accumulated for one sample on dry ice prior to protein extraction.
2. One cap at a time, lyse targets on LCM caps with a lysis buffer compatible with protein downstream analysis, and combine the lysates from each cap for the next protocol step [34].

3.24 LCM Workflow: Retrieval of Target Molecules from LCM Lysates

Column-based extractions are preferred methods for RNA purification, since DNase treatment of RNA can be done during purification, chemical carryover can be completely eliminated, inconsistent and relatively low yield can be amended with target collection approach and modifications to the binding and elution steps of the RNA extraction protocols (*see* **Note 69**). For DNA extraction, both phenol and column-based methods provided reliable results [39–43], however, phenol extraction is a preferred method due to higher and more consistent yields between samples, and recovery of fully intact DNA from samples of high molecular integrity.

The extraction protocols described below have been modified from the manufacturer's protocols for obtaining the highest quality and yields possible from small LCM samples in the various tissue formats. If crude RNA lysate cannot be used in downstream application, we process suitable LCM samples for total RNA extraction with inclusion of the small RNAs/microRNAs fraction. This extraction method provides reproducible results on microarray, RT-PCR, qRT-PCR and RNA-sequencing platform [8, 15, 37, 44]. Simultaneous extraction of RNA and DNA from the same target is possible with the Qiagen ALLPREP DNA/RNA Extraction Kit [45]. However, we experienced lower yields for both RNA and DNA from this kit, and we recommend splitting the lysate for separate purification of RNA and DNA from tiny LCM targets or tissues with low RNA content.

3.25 RNA Extraction Protocol with RNeasy® Micro Kit

1. Work with no more than 12 LCM samples at a time to preserve RNA integrity. Thaw the lysates in wet ice, and equilibrate spin columns to RT. Arrange collection tubes per each step of the protocol. Wipe the inside and lid of the centrifuge with Kimwipe lightly soaked in RNase AWAY, and wipe dry.
2. Keep water from the kit for RNA elution in a thermomixer or heat block at +70 °C. Keep on wet ice DNase I mix and LPA (diluted 1:1 with nuclease-free water). Dilute buffer RPE with ethanol per instructions, and keep it at RT together with vortexed RW1 buffer and 100% ethanol.
3. Transfer tubes from wet ice into a tube rack, and combine lysates of the same sample for a single extraction into a labeled 1.5 ml centrifuge tube.
4. Add 1 μl of LPA, vortex for 30 s and incubate for 5 min at RT.
5. Add 525 μl of 100% ethanol to the lysate and mix well by pipetting up and down 6 times. Do not centrifuge.
6. Apply 525 μl of mixture (including any precipitate) to the spin column, centrifuge for 1 min at 100 × *g*, followed by 30 s at 10,000 × *g*, and transfer the spin column into a new collection tube.
7. Apply the rest of the mixture to the spin column, centrifuge for 1 min at 100 × *g*, followed by 2 min at 10,000 × *g*.
8. Add 350 μl buffer RW1 to the spin column, centrifuge for 30 s at 10,000 × *g*, and transfer the spin column into a new labeled collection tube.
9. Add 80 μl DNase I mix to the center of the spin column membrane and incubate at RT for 15 min.
10. Add 350 μl buffer RW1 to the spin column, centrifuge 30 s at 10,000 × *g*, transfer spin column into a new labeled collection tube.

11. Pipette 500 μl buffer RPE into the spin column, centrifuge at 100 × *g* for 1 min, followed by 30 s at 10,000 × *g*, and transfer the spin column into a new collection tube. Repeat **step 11**.
12. Arrange the tubes in the centrifuge leaving one empty space between tubes, open the caps, centrifuge for 5 min at 16,000 × *g*, and transfer the spin column to a 1.5 ml labeled centrifuge tube for RNA elution.
13. Pipette 17 μl of water in the center of the spin column membrane, centrifuge at 100 × *g* for 30 s, and incubate for 5 min inside the centrifuge.
14. Centrifuge for 1 min at 100 × *g*, followed by 1 min at 10,000 × *g*.
15. Re-apply the eluate back to the spin column membrane, incubate for 5 min, centrifuge for 1 min at 100 × *g*, followed by 3 min at 16,000 × *g*.
16. Proceed with RNA QC or store purified RNA at −80 °C prior to QC.

3.26 RNA Extraction Protocol for PAXgene Tissue miRNA Kit

1. Follow **step 1** from Subheading 3.25.
2. Keep on wet ice DNase I mix, LPA (diluted 1:1 with nuclease-free water) and proteinase K. Dulite buffers TM2 and TM3 per manufacturer's instructions and keep them at RT together with isopropanol, 80% ethanol, and nuclease-free water. Label spin columns, Shredder columns, and 1.5 ml tubes for lysate transfer from Shredder tubes.
3. Follow **step 3** from Subheading 3.25.
4. Add 290 μl of RNase-free water to the lysate, then add 10 μl of PK, vortex for 5 s, incubate the tubes in a Thermomixer for 15 min at 45 °C shaking at 500 × *g*, and spin down.
5. Pipet the lysate directly into a PAXgene Shredder spin column placed in a 2 ml collection tube, and centrifuge for 3 min at 16,000 × *g*.
6. Carefully transfer the entire supernatant of the flow-through fraction to a new 1.5 ml centrifuge tube without disturbing the pellet, and follow **step 4** of Subheading 3.25.
7. Add 675 μl of isopropanol and mix by pipetting up and down 6 times. Do not centrifuge.
8. Pipet 600 μl of mixture into a spin column, centrifuge for 1 min at 10,000 × *g*, transfer the spin column to a new collection tube, and follow **step 7** of Subheading 3.25.
9. Pipet 350 μl Buffer TM2 into the spin column, centrifuge for 30 s at 10,000 × *g*, and transfer spin column to a new collection tube.

10. Add 80 μl DNase I mix in the center of the spin column membrane, incubate at RT for 15 min, centrifuge for 30 s at 10,000 × *g*, transfer spin column to a new collection tube, and keep the collection tube with flow-through.
11. Add 350 μl Buffer TM2 to the flow-through, mix by pipetting up and down 6 times.
12. Pipet the mixture into the spin column, centrifuge for 1 min at 100 × *g*, followed by 30 s at 10,000 × *g*, and transfer the spin column into a new collection tube.
13. Pipet 500 μl Buffer TM3 into the spin column, centrifuge for 1 min at 100 × *g*, followed by 30 s at 10,000 × *g*, and transfer the spin column into a new collection tube.
14. Pipet 500 μl of 80% ethanol into the spin column, centrifuge for 1 min at 100 × *g*, followed by 2 min at 10,000 × *g*, and transfer the spin column into a new collection tube.
15. Follow **step 12** from Subheading 3.25.
16. Pipette 17 μl of Buffer TM4 in the center of the spin column membrane, centrifuge at 100 × *g* for 30 s, and incubate for 5 min inside the centrifuge.
17. Follow **steps 14–16** from Subheading 3.25.

3.27 RNA Extraction Protocol for miRNeasy FFPE Kit

1. Follow **step 1** from Subheading 3.25.
2. Keep water from the kit for RNA elution in a thermomixer or heat block at +70 °C. Keep LPA (diluted 1:1 with nuclease-free water) on wet ice. Dilute buffer RPE per instructions, and keep it at RT together with 100% ethanol.
3. Follow **steps 3** and **4** from Subheading 3.25.
4. Add 1120 μl of 100% ethanol to the lysate and mix well by pipetting up and down 6 times. Do not centrifuge.
5. Apply 700 μl of mixture to the spin column, centrifuge for 1 min at 100 × *g*, followed by 30 s at 10,000 × *g*, transfer the spin column to a new collection tube, and follow **step 7** from Subheading 3.25.
6. Add 500 μl buffer RPE to the spin column, centrifuge 30 s at 10,000 × *g*, transfer the spin column to a new labeled collection tube. Repeat **step 6**.
7. Follow **steps 12–16** from Subheading 3.25 (*see* **Note 69**).

3.28 DNA Extraction Protocol for Animal Tissue DNA Extraction Kit

1. Add 150 μl of TD-S1 to the lysate, mix by pipetting up and down 6 times.
2. Add 175 μl of TD-S2 mix by pipetting up and down 6 times, incubate at RT for 5 min, and centrifuge at 16,000 × *g* for 10 min at RT.

3. Transfer aqueous phase into a new tube, add 1 μl of LPA, mix by vortexing, and add an equal volume (~500 μl) of TD-S4.
4. Mix well by inverting the tube several times; the tubes should be incubated at −20 °C for 2 h to overnight, or can be stored at −80 °C for several months prior to extraction.
5. Centrifuge at 14,000 × *g* for 15 min at 4 °C, then discard the supernatant.
6. Add 700 μl of TD-S5/6 to each tube, vortex, centrifuge at 14,000 × *g* for 5 min at RT, discard the supernatant, and repeat this step.
7. Centrifuge at 16,000 × *g* for 5 min at RT, discard the supernatant, spin down and remove any residual TD-S7, making sure not to pipette the pellet.
8. Air-dry the pellet for 5 min at room temperature, re-constitute in 10–20 μl of a buffer suitable for downstream analysis, incubate at 65 °C for 3 min, and spin down.
9. Store full-length DNA at 4 °C, and FFPE DNA at −20 °C prior to QC.

3.29 DNA Extraction Protocol for Qiagen QIAmp DNA Extraction Kit

1. Dilute Buffers AW1 and AW2 per manufacturer's instructions and prepare elution buffer suitable for downstream analysis.
2. Add 1.0 μl of 10 mg/ml RNase A to each sample, and incubate at 37 °C for 30 min.
3. Add 200 μl Buffer AL, mix by pulse-vortexing for 30 s to yield a homogenous solution.
4. Add 200 μl of 100% ethanol, mix by pipetting up and down 6 times, and incubate for 5 min at RT.
5. Transfer lysate to a spin column, centrifuge at 8000 × *g* for 2 min, transfer the spin column to a new collection tube.
6. Add 500 μl Buffer AW1, centrifuge at 8000 × *g* for 1 min, and transfer the spin column to a new collection tube.
7. Add 500 μl Buffer AW2, centrifuge at 8000 × *g* for 1 min, transfer the spin column to a new collection tube, and centrifuge at 16,000 × *g* for 3 min.
8. Transfer the spin column in 1.5 ml microcentrifuge tube, add 100 μl of elution buffer to the center of the spin column membrane, incubate at RT for 5 min, and centrifuge at 16,000 × *g* for 1 min. Discard the spin column.
9. Concentrate DNA sample as described in **Note 69** without addition of RNaseOut, and follow **step 9** of Subheading 3.28.

3.30 LCM Workflow

QC of RNA and DNA purified from LCM targets.

3.31 RNA QC

1. If the RNA samples were stored at −80 °C, thaw the lysates in wet ice, pulse-vortex, spin down and place in wet ice.
2. Determine RNA concentration and purity by NanoDrop using 1.5 μl of sample (*see* **Note 70**).
3. Determine RNA quality by Agilent Pico Chip using 1 μl of RNA sample at a concentration below 5000 pg/μl (*see* **Note 71**).

3.32 DNA QC

1. If the DNA samples were stored at −20 °C, thaw the lysates in wet ice, pulse-vortex, spin down, and place in wet ice.
2. Determine DNA concentration and purity by NanoDrop using 1.5 μl of DNA sample.
3. Determine DNA quality from frozen and FMFP samples by gel electrophoresis.
4. Determine DNA quality from FFPE samples by Quantifiler (*see* **Note 72**).

3.33 Selected LCM Studies: Large-Scale Study on Archival FFPE Sections Mounted on Glass Slides

LCM technology, providing knowledge about precise localization of genomic/transcriptomic events, is often required for proof of concept in large-scale studies on well-characterized archival clinical samples [40]. Unfortunately, the quality of archival material is not always suitable for successful LCM, especially if the samples are represented by FFPE sections mounted on glass slides. Strong or poor adherence of sections to glass slides is a prevailing problem in such studies. It makes the pickup of dissected target incomplete, or impossible, or lifts a whole section off the slide, thus significantly decreasing the number of data points in the study. Here, we describe a microdissection approach that makes such studies feasible.

1. Inspect slides of the sample set for morphological continuity, and stain one selected slide H&E.
2. Scan H&E slides, and annotate LCM targets on its digital images for LCM reference.
3. Stain the slides with CVAE or LCM H&E stain.
4. Upload annotated images on the Reference Computer monitor.
5. Load three slides in the stage slide holder of an MMI CellCut, bring the image into focus, and reflect the annotations onto the LCM screen image for each of the slides, sequentially.
6. Focus the laser, set laser position, activate the laser, and observe live images for clear laser line (Figs. 7c and 8a). Repeat the procedure for the rest of the slides (*see* **Note 73**).

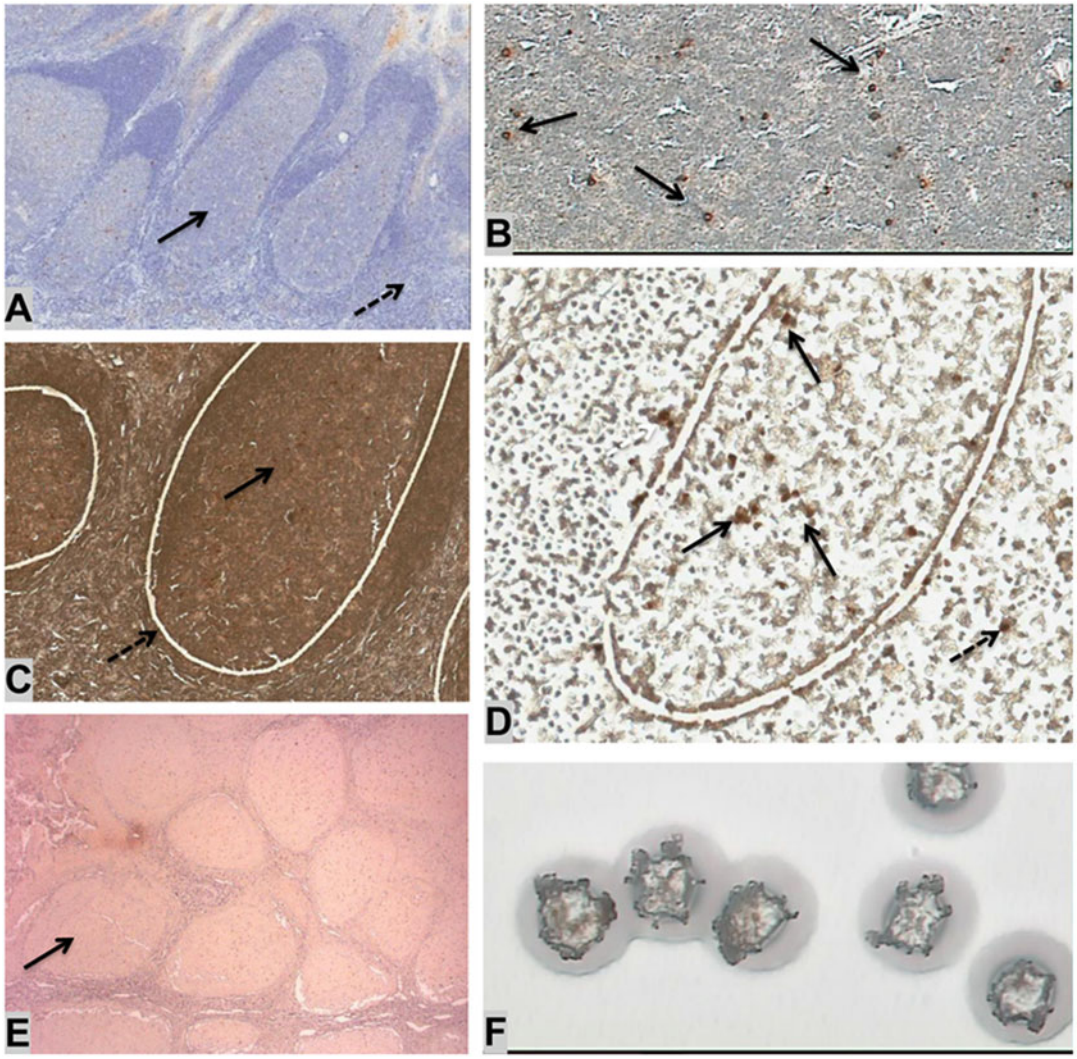

Fig. 7 Laser capture of H2AX positive cells from FFPE sections of human tonsil mounted on glass slides and stained by a routine IHC protocol. (**a**, **c**, **e**) Visualization of routine IHC section of human tonsil: (**a**) View of germinal centers (solid arrow) and intragerminal spaces (dashed arrow) under conventional microscope (Aperio 2× image), (**c**) view of positive cells (solid arrow) and laser path around germinal center (dashed arrow) on Arcturus XT and MMI CellCut screen (4× objective), (**e**) View of germinal centers (arrow) on MMI CellCut screen (section counterstained with diluted (1:50) eosin, instead of hematoxylin, 4× objective). (**b**) Visualization of H2AX positive cells with eosin counterstain on Arctrus XT screen (10× objective). (**d**) Arcturus XT screen view of laser annotated germinal center after trypsin treatment (10× objective): positive cells inside (solid arrows) and outside (dashed arrow) of the germinal center. (**f**) View of H2AX positive cells captures on HS Cap (20× objective)

7. Place a slide under dissecting microscope, apply 100% ethanol until the whole section is soaked, wait for the section to become translucent, and scrape away non-target areas following the laser line (Fig. 8c, f). Blow loose material off

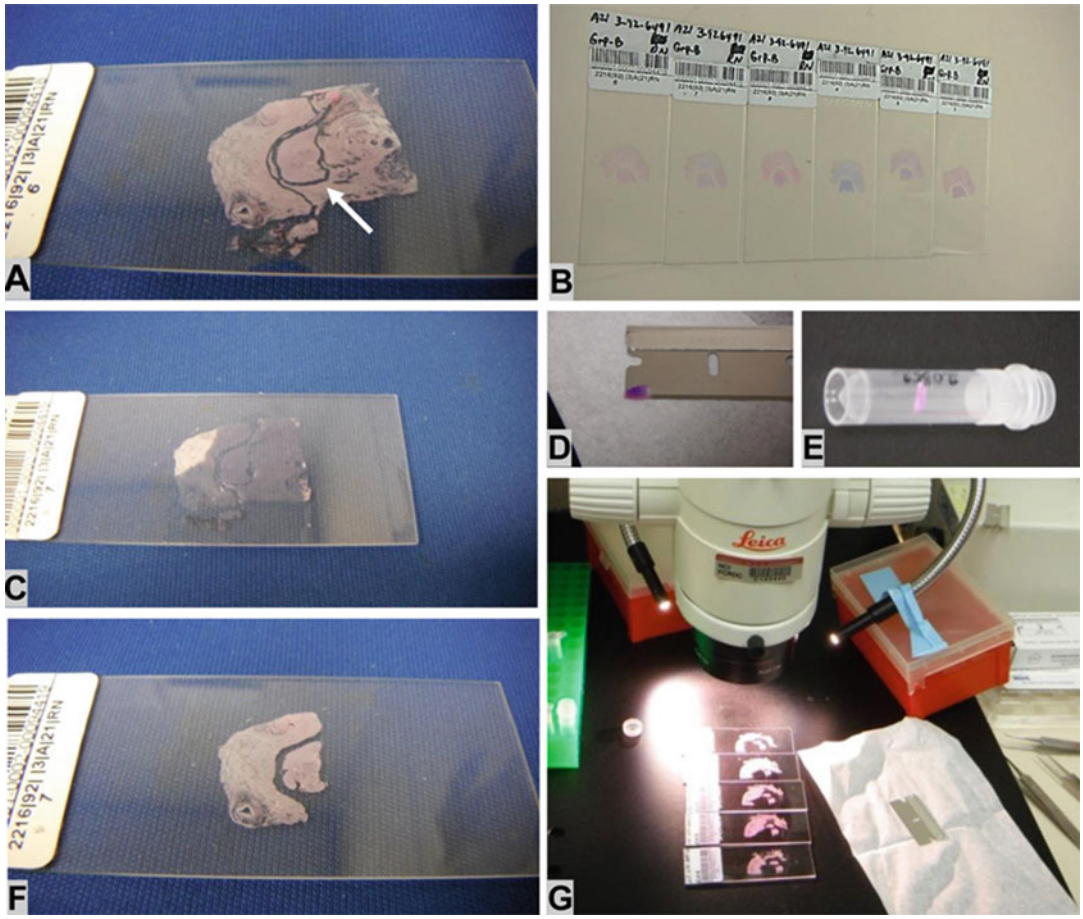

Fig. 8 Laser guided, manual dissection of archival FFPE sections mounted on glass slides. (**a**) View of FFPE section stained with CVAE with annotation lines (arrow) produced by a laser on MMI CellCut. (**b**) Serial slides after the removal of non-targeted tissue from the sections. (**c**) View of a drying section after 100% ethanol application ready for hand dissection. (**d**) Targeted tissue accumulated on the razor blade after scraping. (**e**) View of screw cap tube with targeted tissue. (**f**) View of targeted areas on the slide after the removal of non-targeted tissue. (**g**) View of serial sections aligned for scraping under the dissecting microscope

the slide using compressed air, and place the slide in a desiccator (*see* **Note 74**).

8. Repeat **step 7** for the rest of the slides (Fig. 8b).
9. Arrange the slides with separated targets for simultaneous dissection (Fig. 8g), and apply alcohol to each section.
10. Wait until section is translucent during ethanol evaporation, and immediately scrape one section after another with the same blade (Fig. 8d). Deposit scraped material in the screw cap tube, anchoring the blade along the edge of the tube opening (Fig. 8e). Close the cap and tap the tube on the bench to dislodge the collected material to the bottom of the tube. Open the cap, and let the tissue dry for 15 min.

11. Store dissected samples for RNA at −80 °C and DNA at −20 °C prior to lysis of the completed set for the study (*see* **Note 75**).

3.34 Selected LCM Studies: Dissection of Early Rodent Embryos for Genotyping

Early embryonic lethal phenotypes are common in mouse models of human diseases, and embryo genotyping is required for the analysis of genetic control of development. Genotyping becomes a necessity after a set of studies on serial sections is completed and unusual phenotypes revealed. Often, only one or two glass slides (unstained, H&E or immunostained) with embryos are available for genotyping with no guarantee of successful pick-up of dissected embryos from a glass slide (Fig. 9c). When planning an embryo

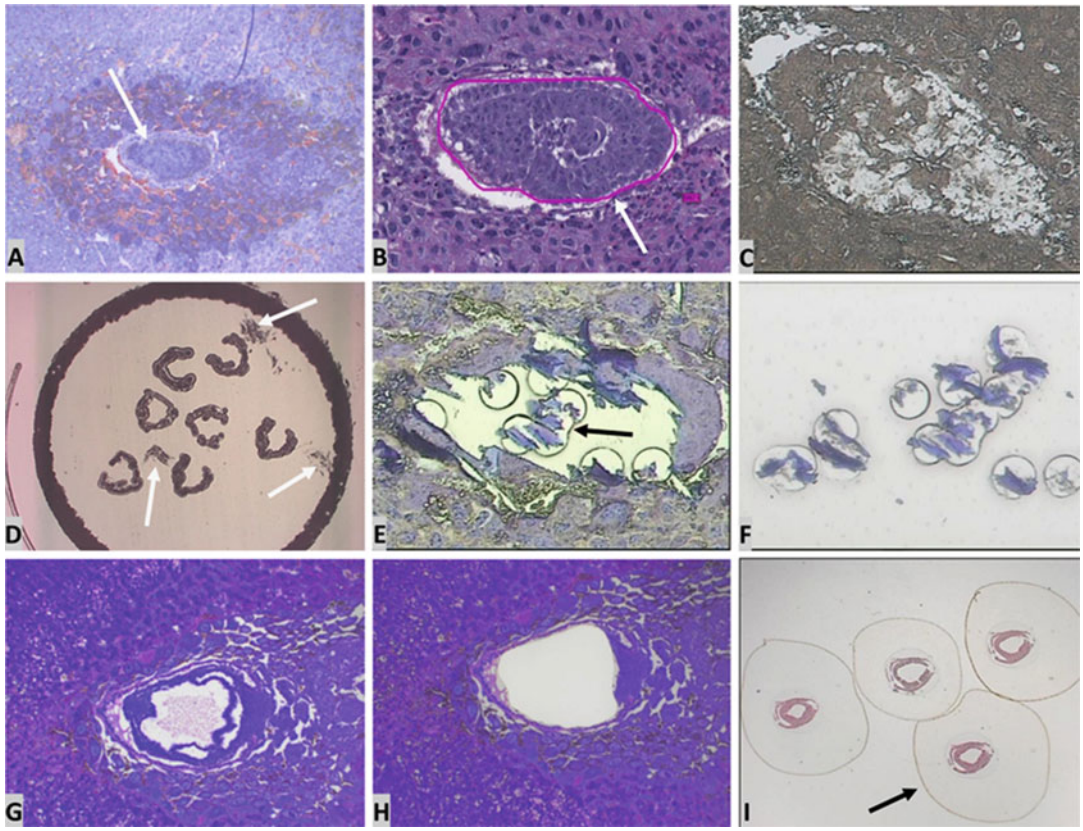

Fig. 9 LCM of early mouse embryos for genotyping on Arcturus XT and MMI CellCut. (**a**) CVAE stained FFPE section of 5 days old embryo (arrow) on MMI Cell Cut screen (4× objective). (**b**) Annotation (arrow) of mouse embryo on LCM reference H&E section (Aperio image, 10×). (**c**) View of a partially collected embryo on the Arcturus XT screen after LCM (CVAE stain, 10× objective). (**d**) View of HS Cap with captured embryos from serial sections and nonspecific contamination (arrows). (**e**, **f**) LCM of mouse embryo on glass slides with strong section adherence: (**e**) laser shots fired over the scraped embryo material (arrow), and the view of scrapped material (**f**) captured on HS Cap (Arcturus XT, 10× objective) (**g**, **h**) Laser cutting of early embryos on frame membrane slides: (**g**) LCM section before dissection and the view of the same section (**h**) after embryo dissection and pick up (MMI CellCut, 10× objective). (**i**) Laser cutting of embryos dissected from decidua: arrow indicates the laser path around the embryo (MMI CellCut, 4× objective)

study with genotyping, consider mounting 1–3 sections designated for genotyping on a frame membrane slide for complete recovery of LCM targets (Fig. 9g, h). Our approach for LCM of early embryos from FFPE sections on glass slides makes embryo genotyping feasible from glass slides [41]. Suitable workflow depends on the type and condition of sample available for LCM. Significant differences in the size of the embryos from the same litter require optimization of PCR reaction for genotyping.

3.35 Rodent Embryos: Unstained Sections on Glass Slides

1. Deparaffinize the section and stain with CVAE protocol (*see* Subheading 3.12, steps **1–7**) or light H&E, coverslip and scan to create an annotated reference LCM image (Fig. 9b) (*see* **Notes 24** and **76**).
2. Incubate the slide in xylene for 2 h, remove the coverslip, and incubate the slide in two changes of xylene for 3 min each.
3. Dry the slide in a fume hood for 5 min and place in a desiccator for 15 min.
4. Dissect the embryo or embryos from several slides (Fig. 9d) on an Arcturus XT with HS Cap following **steps 1–11** from Subheading 3.16.
5. Clean the LCM cap from nonspecific tissue (*see* **Note 4**), cut the film with targets out, and place it in a 0.5 ml centrifuge tube with screw cap.
6. Add 20 μl of lysis buffer from Arcturus Pico Pure DNA extraction Kit and incubate in a Thermomixer for 68 h at 56 °C with shaking at 300 × *g* every 60 min with the addition of 10 μl PK every 24 h of incubation.
7. Incubate the tube at 99 °C for 7 min to inactivate PK, cool the sample for 3 min in wet ice, and transfer the lysate in a new labeled tube.
8. Spin down and store at −20 °C prior to genotyping analysis.

3.36 Rodent Embryos: H&E and Immunostained (IHC) Sections on Glass Slides

In our experience, adherence of H&E and, especially IHC stained sections to the slides is often strong, making it impossible to detach the dissected embryo from the slide. The protocol described below is applicable to such cases.

1. Follow **steps 2** and **3** from Subheading 3.35 (*see* **Note 77**).
2. Place the slide in the stage slide holder of a MMI CellCut, annotate the embryo with a laser path, and follow **step 4** of Subheading 3.35.
3. In case of partial or lack of target pick-up (Fig. 9c), transfer the slide under a conventional dissecting microscope at 1.5–2× magnification.
4. Dispense a drop of 100% ethanol on the embryo, wait until it turns translucent and scrape the embryo using a 27-gauge syringe needle, dipped in 100% ethanol.

5. Move the slide with scraped material into the stage slide holder of Arcturus XT, position an HS Cap on the embryo, fire the laser over scraped material (Fig. 9e, f), and follow **steps 5–8** from Subheading 3.35.

3.37 Rodent Embryos: FMFP and FFPE Blocks

Pathology evaluation of embryo development and phenotype is carried on serial or step sections through the whole embryo. To collect enough LCM material for genotyping, it is advisable to select a section from the sectioning step through the embryo midline.

1. Cut a 7 μm section and mount it in the middle of the frame membrane slide.
2. Stain section with CVAE (Fig. 9g) or light H&E for LCM slides.
3. Scan the stained frame membrane slide to create an annotated image for LCM reference.
4. Dissect the embryo per annotation on a MMI CellCut with 0.5 ml MMI Cap positioned on the membrane over the embryo (Fig. 9g, h) (*see* **Note 78**).
5. Transfer dissected embryos into the tube for lysis following the procedure in **step 5** of Subheading 3.19 for 0.5 ml MMI Caps.
6. Follow **steps 6–8** from Subheading 3.35.

3.38 Selected LCM Studies: Laser Capture of H2AX Positive Cells on FFPE Sections of Human Tonsil for RNA and DNA Retrieval

LCM material retrieved from conventional FFPE IHC and ISH (in-situ hybridization) sections is generally suitable for qPCR analysis. The dissection results for FFPE IHC sections on glass slides are inconsistent due to strong adherence of sections to the slides. Thus, IHC stain of LCM targets should be performed on a membrane allowing a complete removal of dissected material from the slide. The objective of this LCM study was the collection of H2AX positive cells located in germinal centers of lymphoid follicles for DNA and RNA analysis. Heat antigen retrieval as a step of the IHC protocol caused detachment of sections from the membrane of glass membrane slides. Sections on glass slides were not affected by antigen retrieval but developed strong adherence to the slide during the IHC staining. Routine counterstain of IHC sections with hematoxylin resulted in poor visualization on the LCM dissecting screen of PixCell IIe (Fig. 7c). The LCM workflow, designed to overcome mentioned above problems, resulted in successful LCM samples suitable for qPCR.

1. Use ten slides for DNA sample and 20 slides for RNA sample (*see* **Note 79**).
2. Prepare IHC LCM reference slides with hematoxylin counterstain, and scan them to create annotated LCM reference images (Fig. 7a).

3. Stain LCM sections mounted on glass slide with IHC protocol for H2AX detection without hematoxylin counterstain.
4. Counterstain LCM IHC slides with diluted Eosin Y solution (1:50 in 100% ethanol) as follows: 70% ethanol—1 min, 95% ethanol—1 min, Eosin Y—10 s, 100% ethanol (two changes)—30 s each, xylene (2 changes)—3 min each, air-dry—5 min, desiccator—15 min (Fig. 7b).
5. Load the slides on the stage slide holder of an MMI CellCut, draw annotation lines around germinal centers, and activate the laser following **steps 5–11** from Subheading 3.17 (Fig. 7c, e).
6. Transfer the slides to the Slide Humidity Incubation Box, apply 1 ml of 10% trypsin solution (prepared 15 min prior to application) onto each slide, cover the lid, and incubate for 15 min at RT.
7. Rinse the slides in two changes of RNase-free water for 30 s each, followed by 70% ethanol—30 s, 100% ethanol (two changes)—30 s each, xylene (2 changes)—3 min each.
8. Air-dry the slides for 5 min, and transfer to a desiccator for 15 min (Fig. 7d).
9. Dissect the slides (Fig. 7f) following the protocol in Subheading 3.16, **step 1–11** and lyse the targets for RNA following the protocol in Subheading 3.21, steps 1–8. Lyse targets for DNA in Autogen Animal Tissue DNA extraction kit buffer following **steps 3–8** from Subheading 3.22.
10. Extract DNA from DNA lysates following the protocol from Subheading 3.28, steps **1–9**, and RNA from RNA lysates following the protocol from Subheading 3.27, **steps 1–7**.

3.39 Selected LCM Studies: Laser Capture of Insulin Positive Pancreatic Islets on Frozen Repository Samples of Human Pancreas

The objective of this LCM study was the collection of insulin positive islets from repository samples of human pancreas for NanoString RNA analysis. The pilot study showed that RNA yield and RNA quality from LCM targets from frozen sections was higher by smear analysis and NanoString performance compared with FFPE sections in samples with similar RIN numbers. IHC staining for the detection of insulin positive islets completely degraded RNA in frozen samples. RNA was also degraded when we used the UV cutting protocol for target collection from unstained frozen sections. Considering these problems, glass membrane slides were used for section mounting, IHC slides were used for LCM reference, and dissections were performed from sequential sections stained with modified CVA stain, using IR laser on the Arcturus XT, with prior marking of islets positions with UV laser on the MMI CellCut. Visualization of islets was better on the PixCell IIe than on the Arcturus XT. The below LCM workflow, designed to overcome the aforementioned problems, resulted in successful LCM samples suitable for NanoString analysis [24].

1. Check RNA quality in submitted samples by lysing sections on a slide and following the protocol in Subheading 3.25, **steps 1–16** and **steps 12–14** from Subheading 3.4 (*see* **Note 80**).
2. Use ten serial sections for the collection of insulin positive islets for one NanoString sample (*see* **Note 81**).
3. Stain LCM reference slides with IHC protocol for insulin detection substituting hematoxylin with diluted Eosin Y counterstain, as described in **step 4** of Subheading 3.38 (*see* **Note 82**), coverslip slides, and scan them to create annotated LCM reference images (Fig. 10a).
4. Stain three LCM slides following **steps 1**, **3**, and **4** from Subheading 3.13, apply a mixture of CVA and 20 mM Tris buffer (pH 8) at a 3:1 ratio to the section for 20 s (Fig. 10c), drain on Kimwipe and follow **steps 7** and **8** from Subheading 3.13.

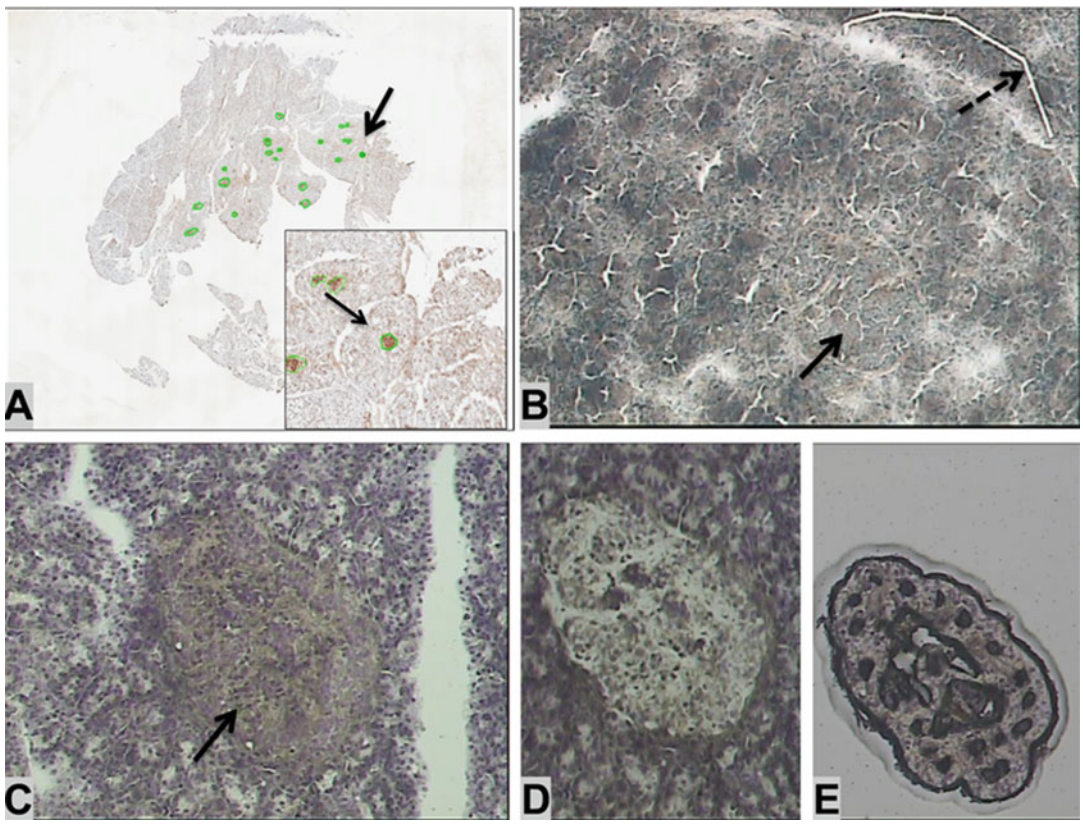

Fig. 10 LCM of insulin positive pancreatic islets on frozen sections of human pancreas. (**a**) IHC LCM reference section (Aperio image, 1×) of human pancreas counterstained with diluted eosin: annotated insulin positive islets (arrows, magnified view in inset). (**b**) View of CVAE stained LCM section on MMI CellCut screen: per reference annotations, positive pancreatic islet (solid arrow) marked by UV laser (dashed arrow) to facilitate IR capture on PixCell IIe. (**c**) Improved visualization of pancreatic islet (arrow) on PixCell IIe after staining with the mix of CVAE and Tris buffer (pH 8). (**d**, **e**) Dissection results: view of the section after target collection (**d**), and the islet captured on HS Cap (**e**)

5. Load three slides on the stage slide holder of the MMI CellCut and mark islets positions, per LCM reference slide, with the short line at a distance of at least 500 μm from the islet to avoid RNA damage by the UV laser. Activate the laser following **steps 5–11** from Subheading 3.17 (Fig. 10b). Store the marked slides in a desiccator during dissection.
6. Transfer a slide to the stage of PixCell IIe, focus the laser per manufacturer's instructions, and capture the marked islets with the HS Cap within 20 min (Fig. 10d, e).
7. Transfer LCM targets into lysis buffer following **step 5** from Subheading 3.19 for *Arcturus LCM Caps* (*see* **Note 61**), and repeat **steps 4–7** with the rest of the slides.
8. Lyse LCM targets for the NanoString sample in 5 μl of buffer RLT with RNaseOut, and remove the films as described in **step 8** from Subheading 3.19.
9. Store samples at −80 °C prior to NanoString analysis.

3.40 Selected LCM Studies: Laser Capture of Tyrosine Hydroxylase (TH) Positive Neurons on Frozen Sections of Mouse Brain

High-quality RNA for microarrays can be retrieved from frozen IHC sections following RNase-free precautions, short direct or indirect IHC protocols with concentrated primary antibodies and fluorescent or alkaline phosphatase RNA-friendly chromogens [46]. Based on our experience, we consider Nanostring and RNA-seq platforms to be more suitable than microarrays for the analysis of IHC LCM material, despite larger input requirements. Optimization of short IHC protocols is time consuming, and the results are dependent on tissue type (RNA stability) and RNA content; IHC protocols include multiple water containing steps detrimental to RNA quality and quantity [46–49]. If the target can be visualized on the LCM dissecting screen with LCM stain, then the IHC stain should be used only for the LCM reference slides, with target collection from sequential sections stained with LCM stain of choice (*see* Subheading 3.39). The objective of this LCM study was the collection of TH positive dopaminergic neurons in ventral tegmental area of mouse brain for RNA microarray analysis. The described below protocol allowed recovery of high-quality RNA (RIN 7) suitable for microarray platform, and can be used as a guide for planning IHC LCM studies on frozen tissue sections. Twenty sections were dissected per LCM sample.

1. Perform mouse necropsy following the guidelines from Subheading 3.2, steps 1–6.
2. Fix brain in 1 ml of DSP fixative for 30 min at RT on a shaker, and embed in OCT as described in **step 7** from Subheading 3.2.
3. Perform RNA QC of embedded tissue as described in Subheading 3.4, **steps 1–3, 4–9** and **11–13** (*see* **Note 83**).

4. Prepare frozen sections mounted on glass membrane slides, and stain an LCM reference slide with a regular IHC protocol.
5. Coverslip and scan the slide to create an annotated LCM reference image.
6. Prepare the following reagents in 50 ml Falcon tubes: (a) Methanol and RNase-free PBS with Triton x100 (0.05%) (**PBSTR**) kept in wet ice, (b) Two changes of 100% ethanol, (c) Two changes of xylene at RT.
7. Transfer slides from dry ice into methanol, incubate for 30 s, and rinse in PBSTR for 15 s with up and down movements.
8. Wipe solution around the tissue with a Kimwipe, place the slide in Slide Humidity Incubation Box, apply HT primary antibody diluted 1:10 in PBSTR with RNaseOUT, and incubate for 5 min.
9. Rinse the slide in PBSTR for 15 s with up and down movement; wipe solution around the tissue with a Kimwipe.
10. Carry out the rest of the protocol in low light conditions.
11. Apply Alexa conjugated secondary antibody diluted 1:25 in PBS with RNaseOut, and incubate for 5 min.
12. Rinse the slides in PBSTR for 15 s with up and down movements, and incubate in two changes of 100% ethanol for 30 s each, followed by two changes of xylene for 1 min each.
13. Air-dry the slides for 3 min and transfer to a desiccator.
14. Set up the Arcturus XT for fluorescence mode per manufacturer's instructions.
15. After 15 min, remove a slide from the desiccator and place in the stage slide holder, annotate the HT positive cells per LCM reference image, and capture with HS Cap within 20 min. Clean the cap from nonspecific contamination (*see* **Note 4**).
16. Transfer LCM targets into 18 μl of RLT lysis buffer with DTT following **step 5** from Subheading 3.19 for *Arcturus LCM Caps* and proceed with lysis and film removal following **steps 6** and **10** from Subheading 3.19.
17. Repeat **steps 7–16** with the rest of the slides.
18. Combine the tubes with lysates for a single RNA extraction and follow the protocol from Subheading 3.25, **steps 1–16**.

4 Notes

1. DSP fixative should be prepared on the day of fixation and stored in wet ice prior to necropsy: Dissolve the reagent in dry DMSO per manufacturer's instructions at 50 mg/ml, aliquot and store at −80 °C for up to 1 year. Prepare fixative for one time use at concentration 1 mg/ml adding the DSP stock

to PBS dropwise, with gentle vortexing (the final solution contains precipitate in suspension). DSP fixed tissues can be embedded in OCT or processed to paraffin.

2. Dissolve 250 mg of Cresyl Violet Acetate powder in 25 ml of 100% ethanol, place on the orbital shaker for 12–24 h, filter for sterile conditions; and age the solution in the refrigerator at +4 °C for at least 6 months for reproducible staining. The solution is usable for 1.5 years.
3. The computer should be connected to the organization's network to make the digital imaging database available during LCM. LCM reference images annotated by the study pathologist should be displayed on the Reference Computer monitor positioned over the dissecting screen of the LCM instrument. This facilitates timely reflection of pathology annotations onto the tissue section on LCM dissecting screen. A wireless mouse should be conveniently located at the LCM station.
4. We found out that Scotch tape cut in 2 × 2 mm squares while in the roll is an excellent mean of cleaning Arcturus LCM caps from nonspecific contamination. Peel a square off the roll with fine tip forceps and lightly touch nonspecific material with the corner of the square on the adhesive side; it permanently sticks to the tape.
5. Standard, reproducible conditions for animal care will reduce variability in gene expression which is greatly affected by heredity, age, general health, physiological condition (e.g., feeding, stage of the reproductive cycle), circadian rhythms, and the season of the year [50–52]. Necropsy is a crucial part of the LCM project since the molecular quality of the collected sample will determine the feasibility of the study and selected downstream applications. Intact samples can be used with any downstream application, providing unbiased results [8, 53, 54].
6. Based on our experience, these conditions proved effective for preservations of RNA integrity in the tissue during sample collection, LCM slide preparation and dissection are fully applicable for DNA and protein LCM projects.
7. Used dissection tools can be cleaned for RNase-free conditions; wipe the blood and tissue debris off with the Kimwipe, rinse in RNase-AWAY, wipe with a new Kimwipe, rinse in two changes of RNase-free water, and wipe the tool dry with Kimwipe.
8. Cross contamination of samples compromises molecular profiling, especially when a fraction of an organ with high RNA content is accidentally introduced in a sample with low RNA content.

9. Blood is rich with RNases and triggers RNA degradation in contaminated samples. [18].
10. RNA degradation in mammalian tissue is a fast process which depends on tissue type and size, temperature, and other factors. Based on RNA degradation data in mammalian cells [17, 55] and our experience with numerous mouse tissues [18], we plan our necropsy protocols for a 6-min completion time after euthanasia. If LCM samples are part of multiple organ collections, prioritize their removal.
11. Samples in cryomolds should be protected from desiccation and accumulation of frost in a resealable plastic bag during −80 °C storage. Both the conditions negatively affect sample morphology and RNA integrity.
12. For five mouse brains, or five livers, or a combination of one whole intestinal tract with two small size tissues per container, fixation for 18 h resulted in good morphology and RNA integrity in FMFP tissues (Table 2, Fig. 2). Shorter fixation times of 3–6 h are suitable for small size organs (e.g., ovaries, pituitary gland, adrenals, early embryos, and tissues trimmed before fixation to 3–5 mm thickness).
13. If a processor designated solely for RNase-free samples is not available, process tissues manually in 1–2 L plastic jars (depending on the number of cassettes) on an orbital shaker.
14. Our processing protocol effectively preserved RNA in mouse tissues (Table 2). LCM procedure resulted in good RNA quality with PAXgene fixed tissues, as well as CVAE LCM staining of DSP and STUMol tissue sections after 1 month storage of paraffin blocks at −20 °C. TheraLin fixative, primarily formulated for the preservation of phosphorylated proteins [28], provided good quality DNA, and integrity of RNA suitable for NanoString analysis and other platforms with the input of nonfull length transcripts.
15. On many occasions, clinical samples were stuffed into 1.8 ml tubes with a screw cap, which made the removal of the samples impossible without thawing, thus compromising their molecular integrity. For such samples, our simple approach of cracking the end of the tube, wrapped in gauze, with the hammer while on dry ice, proved invaluable for preservation of tissue molecular integrity (Fig. 11).
16. RNA*later*® preserved samples are brittle and not suitable for division. Blot tissue with Kimwipes prior to OCT embedding, and cryosection using CryoJane. Such samples have a limited use for LCM due to compromised morphology; only prominent targets can be visualized on LCM dissecting screen (Fig. 2e, f). The feasibility of target visualization should be

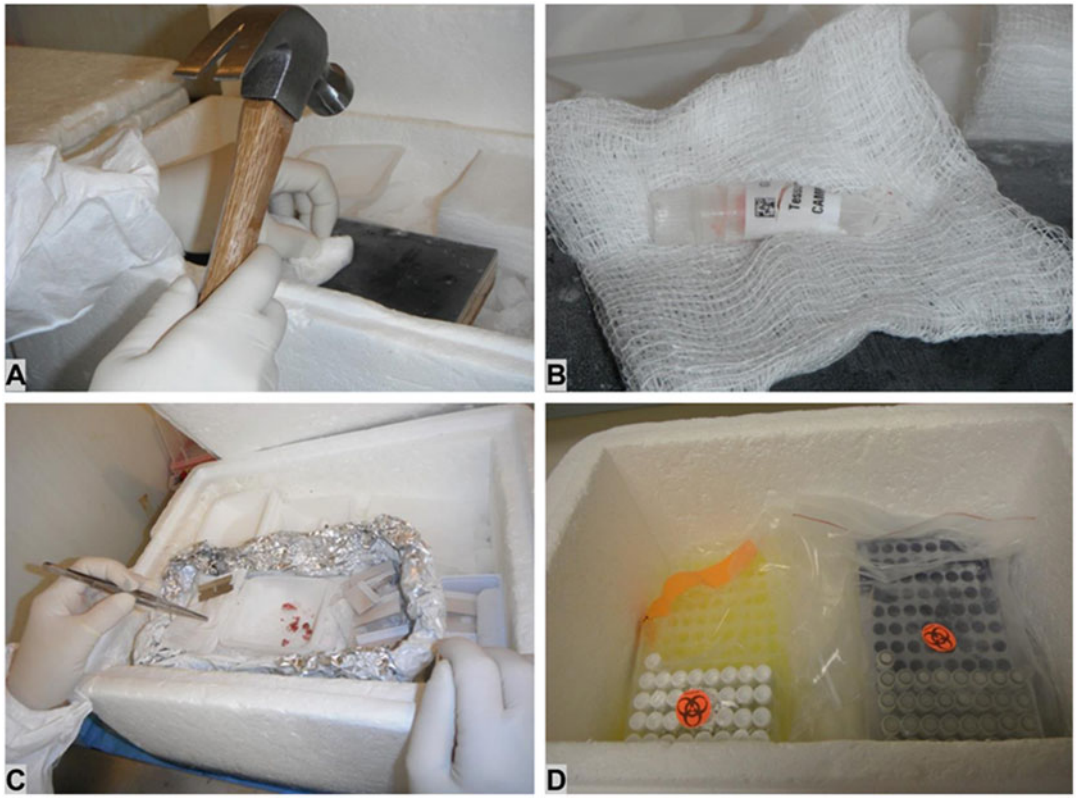

Fig. 11 Sub-sampling of human frozen tissue samples. (**a**) Breaking the tip of the cryotube wrapped in gauze. (**b**) The view of broken tube inside the gauze. (**c**) Tissue division in a weigh boat on dry ice. (**d**) Handling a set of samples in Styrofoam box on dry ice during sub-sampling

confirmed before planning collection of tissues in RNA*later*® for prospective LCM study.

17. It is advisable to group samples of similar quality to secure unbiased results in downstream analysis [56].
18. The surface layer of tissue exposed to atmospheric oxygen and moisture usually has degraded nucleic acids and sub-optimal morphology.
19. This temperature setup is suitable for most tissues. Liver and brain should be cut at −14 or −15 °C, and fatty tissues at temperatures of −20 to −25 °C.
20. OCT and paraffin sections can be mounted on RNase-free membrane LCM slides for the evaluation of both initial RNA quality of a tissue block, and sectioning procedure. This approach is useful for troubleshooting of LCM slide preparation. If only slides are available for the project, the sections should be lysed on the slides, and lysates transferred to tubes for RNA extraction.

21. Slides prepared for H&E can be stained after sectioning or accumulated at −80 °C for batch staining.
22. The use of a new collection tube minimizes the risk of carry-over and shortens the protocol time, thus benefiting RNA preservation.
23. Samples can be stored at −80 °C, thawed on wet ice, vortexed and spun down prior to Nanodrop or Agilent Pico Chip analysis.
24. ***Protocol for H&E stain of OCT sections of fresh frozen tissue***: Air-drying—10 min, 10% neutral buffered formalin—10 min, tap water—5 min, dH_2O—1 min, Hematoxylin-2 (filtered)—2 min, tap water—10 min, 70% ethanol—1 min, 95% ethanol—1 min, Eosin Y—10 s, 100% ethanol—1 min (four changes), xylene—3 min (three changes). If an autostainer is used, load the slides on the stainer in distilled water and start from hematoxylin step after fixation.

    Protocol for H&E stain of OCT sections of DSP-fixed OCT embedded tissue: Rinse in PBS—2 min, Hematoxylin-2-(filtered)—1.5 min, tap water—10 min, 70% ethanol—1 min, 95% ethanol—1 min, Eosin Y—10 s, 100% ethanol—1 min (four changes), xylene—3 min (three changes).

    Stain ***FFPE sections*** on an autostainer using the manufacturer's protocol for H&E. Shortening of the Eosin Y step is required for ***FMFP*** sections due to increased uptake of eosin.

    Protocol for light H&E stain for LCM slides: Dilute hematoxylin and eosin at a 1:10 ratio with RNase-free water and 50% ethanol, respectively, fix OCT section in 70% ethanol for 30 s (paraffin sections should be deparaffinized in two changes of xylene for 5 min each and incubated in two changes of 100% ethanol), apply 1 ml of RNase-free water to a slide for 5 s, drain the slide and repeat this step, drain and apply 200 μl of hematoxylin to the slide for 10 s, drain and apply 1 ml of Bluing reagent for 5 s, incubate the slide in 70% ethanol for 5 s, apply 200 μl of eosin Y and immediately move the slide to 100% ethanol for 5 s, incubate the slide in 2 changes of 100% ethanol for 30 s each, followed by incubation in two changes of xylene for 3 min each. Avoid regular hematoxylin concentration for LCM slides because hematoxylin is inhibitory to Taq polymerase in PCR reactions.
25. In our experience, we designate targets smaller than 100 μm in diameter for IR laser capture because a UV laser significantly affects RNA quality in small targets for most tissues types, with lesser damage in tissues with very stable RNA, like brain and liver.
26. Speed of dissection is a limiting factor for material that can be collected during the established time frame. If the block can be

trimmed close to the LCM target, several sections can be mounted on the same slide (Fig. 4d–f) and dissected with a one-time setup of laser focus and collection device. In case of targets with simple shapes, three slides can be mounted in the stage slide holder for simultaneous dissection.

27. It is important to conserve tissue in LCM blocks which will be needed later for repeated dissection and extraction. Obtaining sections from the blocks containing the largest targets prevents the risk of depleting the tissue prior to main study.
28. Consult literature sources about RNA content in the target tissues taking into consideration the extractions and quantifications methods used.
29. With the progression of serial cutting, the size and shape of targets significantly change, moreover, some targets disappear, and new targets appear on the section. If more sections are needed for the continuation of a pilot, it is important to have a reference H&E of the new area to evaluate a number of slides for repeated dissections.
30. If longer dissections could be beneficial for the project, test the effect of longer dissection times on RNA integrity in LCM targets, and use the established time frame for the main study.
31. A range of tissues containing collagen, fat, cartilage, bone and delicate structures (e.g., mouse normal ovarian epithelium) as well as plant tissues are not suitable for routine serial cryosectioning; random loss of material during cryotomy and staining, poor morphology on the LCM dissecting screen, and excessive contamination of LCM target with nonspecific tissue during the collection of dissected area is common. Based on our experience, CryoJane Tape-Transfer System® is an optimal solution for such cases, using either commercial glass CryoJane slides (Fig. 6) or membrane slides manually coated with CryoJane adhesives (Fig. 12) [21]. We think that glass CryoJane slides should be used for all the tissues intended for IR laser capture microdissection, because it provides a foolproof cryosectioning procedure for any type of tissue and maximizes the use of unique samples. Adopting the CryoJane technique to membrane slides increases chances of making challenging LCM projects feasible (Fig. 12).
32. CVAE stain is our first choice for LCM projects. We obtained excellent visualization of LCM targets on dissecting screen of MMI CellCut (Fig. 13) for a variety of human and rodent tissues. However, target visualization on archival paraffin sections mounted on glass slides, on some occasion, is not optimal with CVAE; modified H&E stain can give better results, instead. OCT sections for IR laser capture on Arcturus XT or PixCell IIe we stain with MethylGreen because all the three

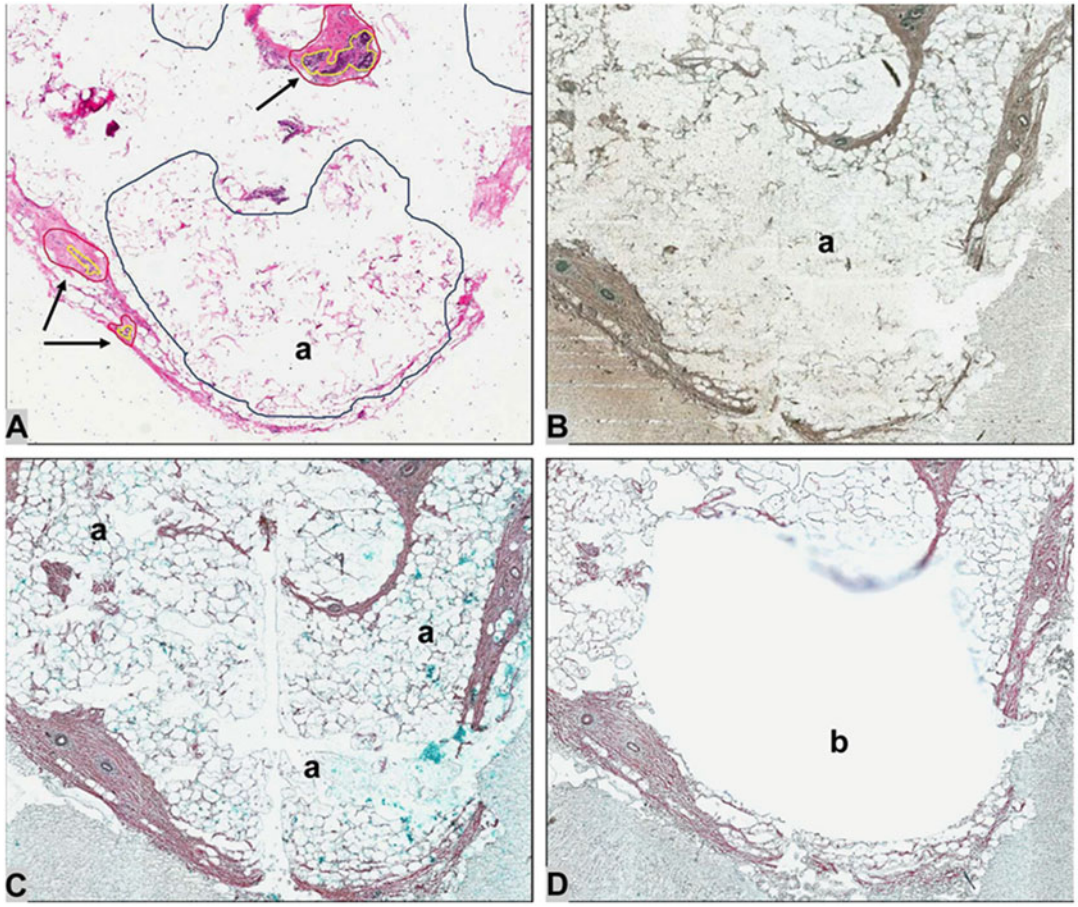

Fig. 12 UV laser cutting on membrane CryoJane slides: OCT sections of human breast biopsy. (**a**) LCM reference H&E of human breast biopsy (glass CryoJane slide) with annotated LCM targets: intact ductal epithelial and fibroblast (arrows) and partially lost fat (a) (Aperio image, 4×). (**b**) View of biopsy section (glass CryoJane slide, Methyl Green stain) on LCM dissecting screen: fat (a) is lost during section mounting. (**c**) View of biopsy section on manually coated membrane CryoJane slide (CVAE stain) on LCM dissecting screen: fat (a) is intact throughout the tissue section. (**d**) View of the same section as in (**c**) after UV laser cutting and target removal (b). (**b**)–(**d**): MMI CellCutPlus at 4× magnification

stains give the same appearance on the dissecting screen but MethylGreen is a shortest stain providing greater RNA yield and integrity. For this reason, we routinely use Methyl Green in OCT removal step (instead of water) followed by CVAE [15, 21].

33. Two extra slides are used as test slides for the setup of laser and camera parameters. In case of CryoJane technique, all the sections are mounted on ½ × type CryoJane slides, and every sixth slide is separated from the batch for future H&E staining.
34. Section thickness should be established in a pilot study. It depends on the tissue RNA/DNA/protein content, tissue

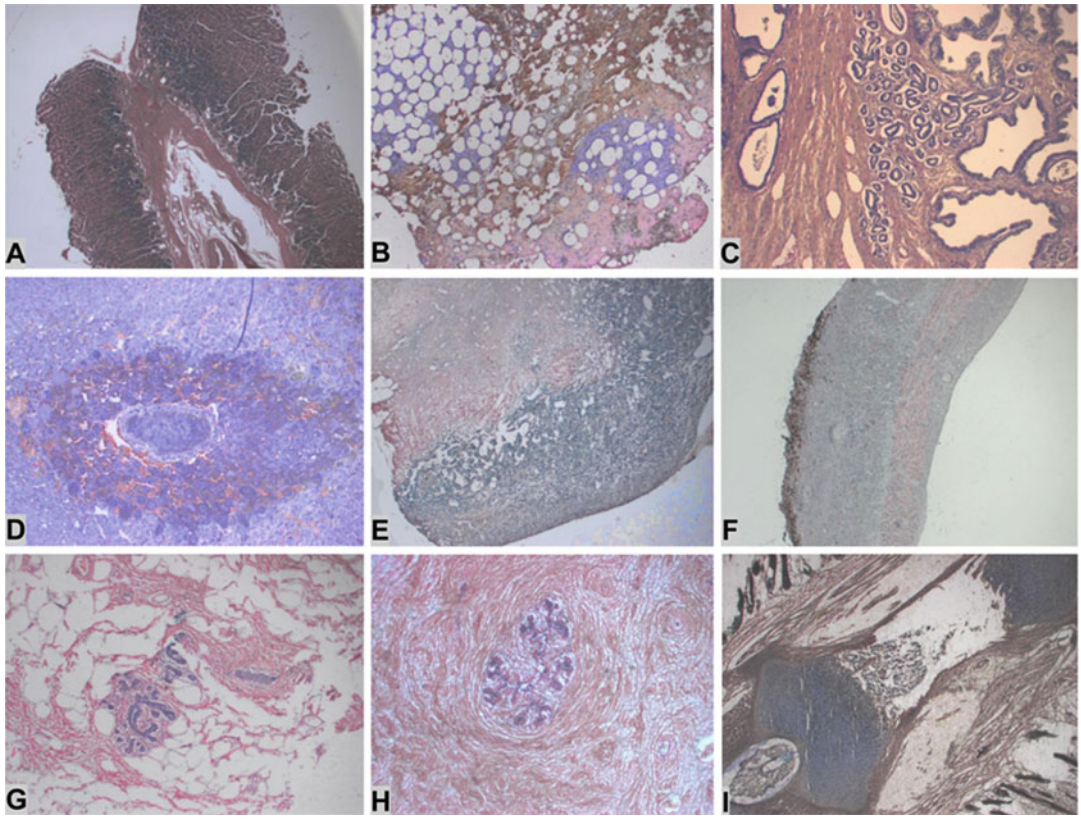

Fig. 13 Target visualization on MMI CellCut dissecting screen: CVAE stained sections mounted on membrane and CryoJane slides. (**a**) Paxgene fixed human stomach biopsy (LCM target-mucosal glands and muscularis propria,). (**b**, **c**) FFPE sections of human bone marrow and prostate biopsy, respectively (LCM target-lesions). (**d**) FFPE section of mouse embryo (LCM task-separation of embryo from decidua). (**e**, **f**) Frozen OCT embedded mouse tumors: mammary gland (LCM target-carcinoma) and skin (LCM target-melanoma), respectively. (**g**–**i**) Frozen OCT embedded tissues on CryoJane slides: human breast biopsy on membrane (**g**) and glass (**h**) CryoJane slide (LCM targets-ductal epithelium, fibroblast and fat), dog tail on a glass CryoJane slide (**i**) (LCM targets-vertebra, muscle). MMI CellCut magnification 4× (**a**–**c**, **g**), 10× (**d**–**f**, **h**, **i**). (**a**, **c**, **i**) MMI Cap is in *up* position, (**b**, **d**–**h**) MMI Cap is in *down* position

and target type, and visualization on the dissecting screen with the stain of choice.

35. Equilibration of OCT block to cryostat temperature is crucial for the morphological quality of the section. The significant change in temperature between storage and cryostat chamber could be detrimental to the tissue RNA integrity (especially true for RNase-rich tissues). For this reason, minimize the exposure of the OCT block to the cryostat temperature by preparing for sectioning only one block at a time.
36. Upon our experience, 2-week storage prior to microdissection is safe for RNA integrity in most tissues. We prefer to store LCM slides in plastic Five-Slide Mailers instead of 25 or 50 slot

slide boxes. It allows handling one container at a time for dissection, keeping the rest of the slides at constant −80 °C temperature for preservation of RNA integrity.

37. Exposure to UV light makes membrane slides RNase-free and facilitates better adherence of frozen sections to the membrane during mounting. Slides should be treated with UV shortly prior to sectioning. Upon our experience, the treated slides retain their adhesive properties within 2 h of treatment. Repeated exposure of slides to UV does not affect the membrane or slide.
38. Use lab marker during sectioning and affix the label to dried slides because not all types of markers are resistant to xylene.
39. Soaking time is tissue and fixative dependent, and it affects section adherence to the membrane and appearance of the stain on the dissecting screen. On average, soaking time for FFPE and alcohol fixed tissue blocks is 20–30 min. DSP, STUMol, and PAXgene tissue blocks should be soaked for 10 min, sectioned and returned into the soaking boat for another 10 min for the next route of sectioning. Soaking intervals for TheraLin tissues should be 5 min. This procedure is more laborious compared to the long soaking time, but consistently maintains molecular integrity in LCM sections.
40. This step minimizes slide to slide variation in RNA integrity in stained LCM slides. Two slides can be stained at a time in a "back-to-back arrangement" with labels facing each other.
41. Staining duration can vary from 5 to 30 s, the latter, on average, provides for good visualization. Duration of staining with CVAE should be determined in a pilot study, since it depends on tissue type and the age of CVA stock solution, as well as section thickness and target visualization on LCM dissecting screen.
42. Drying in a desiccator improves laser focusing and efficiency of dissection. Slides with sections of mouse primary tumors, lung metastasis, and normal mammary gland and lungs did not show any sign of RNA degradation during the 5-h storage in a desiccator prior to dissection [15].
43. The invention of membrane slides greatly improved the outcome of LCM technique originally based on Arcturus LCM caps and utilization of glass slides for both OCT and paraffin embedded tissues, with unpredictable strength of section adherence to the slide [1, 2, 18, 20], necessity of pick-up efficiency test on different types of glass slides [18] and intrinsic problem of nonspecific contamination of collected target with tissue adjacent to a melted spot on the cap. The degree of contamination, if any, is tissue type dependent, and the removal of nonspecific tissue from the cap is necessary [18, 20, 21].

The further improvement came with the CryoJane technology for OCT sections; the use of CryoJane adhesive slides guaranties a complete and often clean pick-up of laser-captured targets with Arcturus LCM caps. Arcturus XT and PixCell IIe instruments are equally suitable to use with CryoJane slides.

44. HS Cap has smaller dissection area with cleaner target pick-up, and it is more suitable for the dissection of small targets. Macro Cap has larger dissection area but catches more nonspecific material from the section. Nonspecific contamination of LCM targets is a larger problem with such tissues as skin, lung, stomach, small intestine, colon, mammary gland, necrotic tumors, and low quality sections of uneven thickness.
45. Average dissection time which guarantees high-quality RNA and protein in LCM targets is 20 min for OCT tissues, 30 min for FMFP tissues, 1 h for DNA from OCT, and up to 2 h for DNA, RNA and proteins from FFPE tissues. Set a timer for 2 min shorter than the dissection time; on average, it takes 2 min to get the dissected cells into the lysis buffer.
46. The software automatically adjusts the spot size to the magnification selected for dissection, which benefits the dissection precision while capturing targets close by size to the dimensions of the individual cells (e.g., normal ovarian epithelium—a single-cell layer covering the ovary). The spots produced by software along the draw line are identical; however, the width of epithelial layer varies as well as the shape of the ovary. Upon our experience, the draw line should be set the way that the prospective melting ring covers the epithelial cell just touching the ovary. It takes several attempts to draw an annotation line for dissection to ensure that polymer melting rings do not extend into the ovary beyond the ovarian epithelium. However, nonspecific contamination from the adjacent ovarian tissue is not a rare occasion, and must be removed using the Scotch tape squares (*see* **Note 4**). This procedure does not affect RNA quality in LCM target (Fig. 6e, f).
47. MMI CellCut is our preferred instrument for laser cutting projects due to the excellent visualization of tissue on the dissecting screen (Fig. 13) and flexibility with collection and lysis of dissected targets.
48. For better section adherence, membrane slides can be treated with 8% APES solution (Sigma) in dry acetone [22, 34]. Alternatively, loose non-target tissue adjacent to the target should be dissected and removed prior to the annotation and dissection of the target areas.
49. For RNase-free conditions, incubate the slides in RNase AWAY for 2 min, rinse in three changes of RNase-free water for 3 min each, and dry at 37 °C protected from contamination.

50. For a metal frame slide to be positioned tightly in the slide holder, a glass support slide should be the same width or narrower than the frame slide. The section, sandwiched between the membrane and glass support slide, is protected from contamination during dissection.
51. Use Macro Cap for laser cutting on Arcturus XT. Collection caps with both instruments dramatically improve the tissue visualization on the dissecting screen, and are mandatory during target annotation with *Draw* function of the software. However, the cutting can be done without the cap, which is useful for timely collection of large targets, and for tailoring dissection approach to the input requirements of downstream applications.
52. To speed up the drawing, use 2× or 4×-objective to draw around large targets on LCM screen; change to 10×-objective for dissections. The calibration for precise match of the drawing lines between objectives should be done on a test slide.
53. If the target remains on the slide after pick-up attempt, inspect the target for spots of incomplete cut, draw the line in these spots and cut for full detachment. Cut the large targets with the cap off the membrane. Cut and collect small targets with the cap in the down position, on the membrane, since static often displaces small cutouts. Upon our experience with both Arcturus XT and MMI CellCut, it is safer to draw and pick up one target at a time, instead of drawing several targets simultaneously for automated cut/pick up function of the instrument. This approach is especially applicable to unique samples with a limited number of targets, since the up and down movements of collection cap, and metal frame imperfections tend to flex the membrane during dissection, thus creating misalignment between the draw line and laser path during sequential cutting and picking of the target.
54. For paraffin sections, the support slide can be left in the slide holder for the entire batch of slides; it does not affect molecular quality and allows for throughput dissection of serial sections [34].
55. We had mixed results with storing LCM samples at −80 °C as cutouts prior to lysis but consistently retrieved high-quality RNA when samples were stored as lysates.
56. For downstream analysis requiring extracted RNA (microarray, RNA-seq, Fluidigm) we use RNeasy Micro Kit with buffer RLT prepared with β-mercaptoethanol (10 μl BM to 1000 μl of buffer RLT) for fresh frozen tissues and with β-mercaptoethanol and DTT (3.86 mg of DTT per 1 ml of RLT-β-mercaptoethanol buffer) for DSP fixed and OCT embedded tissues. We add DTT to Buffer TM1 (PAXgene kit) at the same concentration for DSP fixed paraffin-embedded tissues.

57. The cutouts from the batch of LCM slides can be accumulated in a tube with 350 μl of lysis buffer in wet ice until the completion of LCM session. The time frame for accumulation should be determined in a pilot study since RNA stability is tissue dependent. In our experience, RNA in targets from mouse liver remained stable for 4 h [21], and in mouse mammary gland carcinoma for 2.5 h [15]. However, this approach was not suitable for mouse normal ovarian epithelium; RNA quality was consistently high only with separate lysis of targets from each dissected slide.
58. We found that it was difficult to submerge films peeled off the Arcturus caps in volumes below 20 μl. If more than 15 slides have to be dissected for single extraction, modify the extraction protocol for the increased input volume, or use accumulation approach if suitable. Also, the extraction protocol should be scaled up for increased starting volume of RNA lysate for RNase-rich tissues because larger volumes of lysis buffer per slide are beneficial for preservation of RNA integrity in such samples.
59. Place an RNase-free glass slide on the top of the membrane slide secured in the stage slide holder (Fig. 5j, k), and transfer a three-slide assembly under the dissecting microscope. Carefully remove the top slide, gently detach the cutouts from the membrane, collect them in a lump and place into lysis buffer. Dissect three slides simultaneously and place all the targets in one tube with 350 μl of lysis buffer, if the amount of RNA from three slides is sufficient for extraction.
60. As a rule, the retrieval of sufficient amount of RNA for analysis from small LCM targets requires accumulation of targets from multiple LCM slides. Thus, lysates from individual slides should be combined for single RNA extraction, or cutouts from individual slides should be accumulated in one tube with lysis buffer during LCM session.
61. Slice target area in multiple pieces for low volume (5–10 μl) lysis procedure (as for NanoString platform) to completely submerge all the targets in lysis buffer.
62. Check the cap for the presence of LCM targets. If some targets remained on the cap, repeat the procedure re-using ethanol in the tube.
63. Small volume collection approach was successfully used with Arcturus HS and MMI Caps for NanoString sample collection [15, 24, 34].
64. Small pieces of membrane plug the tip causing pipetting inaccuracy, which is crucial for applications based on 1–4 μl input (e.g., NanoString). Also, membranes stuck to the filter of extraction column during binding step contribute to RNA/DNA loss during extraction.

65. Use 20 μl pipette tips for PCR tubes, and 200 μl tips for 0.5 ml centrifuge tubes. Transfer lysate to the filter/tube assembly and centrifuge the assembly at 16,000 × *g* for 30 s, making sure that the lysate completely passed through the filter. Using a pipette tip, collect membranes/films from the bottom of the tube in a lump, transfer to the filter, centrifuge the tube until they are dry, and discard the filter.
66. We consistently recover high-quality RNA from lysates stored up to 2 months.
67. For NanoString sample prepare lysis buffer containing 2 μl of buffer RLT with β-mercaptoethanol from RNeasy Micro kit, 3.8 μl of RNase-free water, 1.0 μl PK from FFPE RNeasy kit, and 0.3 μl of RNaseOut. Place the PCR tube with LCM sample in a container with screw cap to avoid evaporation of lysis buffer during 68 h incubation, filter out the membranes/films, and store the tube at −80 °C prior to NanoString analysis [34].
68. Arcturus kit is used for applications with crude lysate input, however, DNA can be purified from lysate using column or phenol-based extraction methods.
69. All the methods have their pros and cons, so the proper extraction method must be chosen for downstream analysis. Phenol-based extraction usually has the highest and most consistent yields, but phenol carry over can affect some analysis platforms. Also, if DNase treatment is required with phenol method, it is performed after the RNA purification, resulting in decreased RNA yield. There is approximately 40% loss of nucleic acids every time it is purified. Most bead base methods have the lowest yields and, thus, not suitable for LCM samples. RNA content in FFPE LCM samples is significantly lower than in frozen samples; increased elution volume followed by sample concentration can be used to maximize RNA recovery. Modify elution step of miRNA FFPE protocol as follows: (a) Pipette 100 μl of water in the center of the spin column membrane, centrifuge at 100 × *g* for 30 s, incubate for 5 min inside the centrifuge at RT, and follow **steps 14** and **15** from Subheading 3.25, (b) Add 4 μl of RNaseOut, vortex for 5 s, spin down, and place a mark for a volume of 10 μl on the tube, (c) Put the tube with the open cap into a SpeedVac, disable the heat and process until the volume in the tube goes down to the mark, (d) Measure RNA concentration, and repeat sample concentration as needed.
70. NanoDrop measurements below 4 ng/μl are not reliable; RNA concentration for such samples should be estimated using Agilent 2100 Bioanalyzer PicoChip.
71. Agilent Pico Chip is the fast, suitable, and convenient method of RNA QC in LCM samples known for their low RNA

content. Samples with RIN 7 or greater can be used for almost all downstream analyses, RIN below 5.0 may be limited to PCR based assays or NanoString platform. FFPE samples are usually highly degraded, and RIN is not a good determination of their RNA quality. Use Agilent's smear analysis feature in the 2100 Expert software to determine the percentage of RNA fragments in each sample. For example, samples with 50% of transcripts >300 bases provide valid results for Illumina RNA sequencing, and for NanoString, gene profiling data comparable with frozen samples.

72. The percentage of amplifiable DNA from FFPE samples is sample dependent and greatly affected by type and time of fixation, parameters of processing and embedding in paraffin, conditions and length of storage. Upon our experience [40, 42, 57], a PCR-based assay such as Quantifiler is a better determinant of sample performance in downstream analysis compared to fluorometric methods of DNA quality assessment, with the added benefit of internal positive control, which detects the presence of PCR inhibitors in DNA samples.
73. If the size of target areas on the section is not suitable for laser cutting, transfer the section on glass membrane slide for laser capture, according to the method described in ref. 34.
74. If the target adheres poorly to the slide, do not use compressed air. Instead, clean the nonspecific debris with scotch tape squares (*see* **Note 4**).
75. The described approach was successfully used for the large-scale study of prostate cancer based on analysis of LCM targets from archival slides [40].
76. CVAE is a preferred stain, though, on occasions when it does not provide suitable visualization of the embryo on LCM screen, light H&E stain should be used. Use the slide for dissections if annotations are not necessary for LCM. Alternatively, annotations can be done with the laser pass on MMI CellCut.
77. We noted that samples collected from intensely stained H&E slides perform in PCR reaction worse than samples from unstained slides. We remove the stain by the following method: (a) Rehydrate the slide in 100% ethanol, followed by 95 and 70% ethanol for 1 min each, (b) rinse in distilled water for 2 min and place into 200 ml of pH 6 citrate buffer (c) incubate in the microwave for 10 min at 100 °C, (d) Rinse in two changes of distilled water for 2 min each, dehydrate to 100% ethanol and stain with CVAE for LCM.
78. If the embryos were out of the decidua, draw a line for laser path far from the embryo (Fig. 9i) to minimize the UV damage to the tissue.

79. The number of slides required for a single extraction and the number of slides for simultaneous staining should be determined in a pilot study. The number of slides stained as a batch for LCM session depends on the speed of dissection.
80. Human repositories rarely release the blocks but, instead, prepare sections upon request; RNA QC should be done from the section scrape from the slide. On average, 30% of samples from repository had completely degraded RNA. So, it is advisable to increase number of samples in the study to have a flexibility of excluding low-quality samples, and grouping samples of the same RNA quality for downstream analysis.
81. On average, 7–8 slides (out of ten) satisfied NanoString input requirements, resulting in collection of 40–100 islets per sample. However, pick-up efficiency of captured targets and number of positive islets varied from slide to slide.
82. This counterstain gives visualization of morphological details on Reference Computer monitor comparable to the detail in CVAE stained section on both MMI CellCut and PixCell IIe dissecting screens.
83. Our sample acquisition protocol of mouse brain resulted in RINs range 9–9.8.

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Chapter 4

Protocol for the Analysis of Laser Capture Microdissected Fresh-Frozen Tissue Homogenates by Silver-Stained 1D SDS-PAGE

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Abstract

The heterogeneity present in solid tumors adds significant difficulty to scientific analysis and improved understanding. Fundamentally, solid tumor formation consists of cancer cells proper along with stromal elements. The burgeoning malignant process is dependent upon modified stromal elements. Collectively, the stroma forms an essential microenvironment, which is indispensable for the survival and growth of the malignant neoplasm. This cellular heterogeneity makes molecular profiling of solid tumors via mass spectrometry (MS)-based proteomics a daunting task. Laser capture microdissection (LCM) is commonly used to obtain distinct histological cell types (e.g., tumor parenchymal cells, stromal cells) from tumor tissue and attempt to address the tumor heterogeneity interference with downstream liquid chromatography (LC) MS analysis. To provide optimal LC-MS analysis of micro-scale and/or nano-scale tissue sections, we modified and optimized a silver-stained one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1D-SDS-PAGE) protocol for the LC-MS analysis of LCM-procured fresh-frozen tissue specimens. Presented is a detailed in-gel digestion protocol adjusted specifically to maximize the proteome coverage of amount-limited LCM samples, and facilitate in-depth molecular profiling. Following LCM, targeted tissue sections are further fractionated using silver-stained 1D-SDS-PAGE to resolve and visualize tissue proteins prior to in-gel digestion and subsequent LC-MS analysis.

Key words Laser capture microdissection (LCM), Fresh frozen tissue, 1D SDS-PAGE, Silver stain, Solid tumor heterogeneity, Parenchyma, Stroma, Liquid chromatography mass spectrometry (LC-MS)-based proteomics, Biomarker discovery, Drug discovery, Tumor microenvironment

1 Introduction

Molecular profiling of a patient's tumor has the capability to provide insight into the cancer-induced changes that may ultimately facilitate biomarker and/or drug target discovery and lead to individualized therapeutic interventions [1, 2]. At the cellular level, solid tumors exhibit a high level of heterogeneity. Solid tumors have a distinct structure that mimics normal tissue and comprises two distinct but independent compartments: the parenchyma

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(neoplastic cells) and the stroma, which the neoplastic cells induce, and in which they are dispersed [3]. While the majority of pharmaceuticals target the parenchyma, stromal cells are becoming increasingly recognized as critical therapeutic targets, for instance: (1) targeting a fibrotic extracellular matrix and cancer-associated fibroblasts, (2) interfering with growth factor or cytokine-mediated signaling (e.g., TGF-β), (3) anti-angiogenesis, and (4) neutralizing inflammatory responses [4, 5]. More recently, cancer immunotherapy focused on blocking immune checkpoints with monoclonal antibodies has emerged as an extremely exciting strategy to achieve durable tumor shrinkage or even cure in some instances, in several tumor types, and involves targeting a variety of cells outside of the tumor proper [6].

Although the data obtained from cancer cells cultured in vitro are quite important, it lacks information regarding the tumor microenvironment and limits the translational aspect of these results, and additionally due to the biochemical derrangements related to in-vito adaptations [7]. PDX models may recapitulate the tumor proper and aspects of a microenvironment to some degree. Certainly, the ability to deliberately dissect the various aspects of tumor tissue for direct proteomic analysis is critical for a better understanding of tumor biology and novel drug discovery [8].

Like most technologies, established methods designed to study various tumor tissue types have evolved over time. Macrodissection methods [9] have given way to more accurate microdissection protocols [10]. However, few can argue that perhaps the most precise technique designed to conquer the challenges and biases associated with studying tumor tissue heterogeneity is LCM [11–13].

LCM is a recognized technique designed to overcome the heterogeneity impediment associated with various tumor tissue types. LCM enables the isolation of homogenous cellular populations from heterogeneous cellular mixtures of tumor tissue under microscopic visualization. Previously, we have shown that LCM coupled with LC-MS can effectively be used for direct solution-based proteomic profiling of thin fresh-frozen tissue sections [14]. However, LCM specimens containing less than 10,000 cells require an additional separation step to obtain optimal proteome coverage. In this context, gel-based separations have been routinely used to resolve amount-limited complex protein mixtures [15, 16].

Here described is a silver-stained 1D-SDS-PAGE protocol, modified and optimized specifically for the molecular profiling of amount-limited LCM specimens using MS-based proteomics (Fig. 1). The detailed preparation steps concerning LCM tissue have been previously described by our laboratory [17, 18]. This protocol is an addition to our previously described LC-MS methods for the analysis of LCM-specimens. It is also amenable to other biological samples, which may be limited in protein concentration, and/or volume.

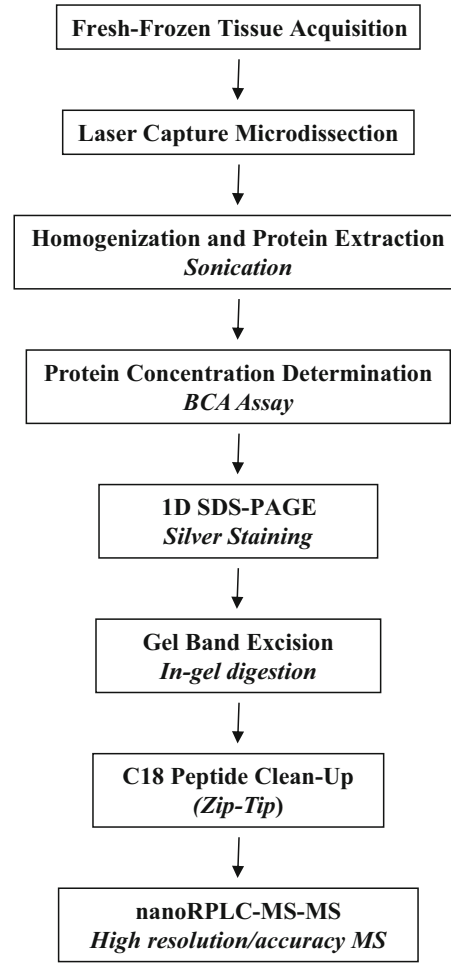

Fig. 1 LCM-based fresh frozen tissue preparation workflow. LCM fresh frozen tissue preparation, silver staining and sample processing workflow is presented

2 Materials

2.1 General Reagents/Equipment

1. LC-MS CHROMASOLV® Water. (Sigma Aldrich)
2. Methanol, LC-MS grade. (Sigma Aldrich)
3. Ethanol, HPLC grade. (Sigma Aldrich)
4. Acetic Acid, HPLC grade. (Sigma Aldrich)
5. Acetonitrile (ACN), LC-MS grade. (Sigma Aldrich)
6. Trifluoroacetic acid (TFA), LC-MS grade. (Sigma Aldrich)
7. Formic Acid (FA), LC-MS grade. (Sigma Aldrich)
8. Single channel manual pipettes: 0.1–2 μL, 0.5–10 μL, 2–20 μL, 10–100 μL, 20–200 μL, 100–1000 μL.

9. Racked pipette tips: 10 μL, 250 μL, 1000 μL.
10. Safe-lock Eppendorf tubes: 0.5 mL, 1.5 mL, 2.0 mL. (VWR)
11. Drummond Portable Pipet-Aid. (VWR)
12. Glass serological pipettes: 2 mL, 5 mL, 10 mL, 25 mL, 50 mL. Vortex mixer. (VWR)
13. Refrigerated microcentrifuge 20R. (VWR)
14. Platform rocker 200. (VWR)
15. −80 °C freezer.

2.2 Fresh Frozen Tissue Acquisition and Sectioning

1. Fresh-frozen tissue.
2. Liquid nitrogen.
3. TISSUE-Tek O.C.T. cryostat mounting medium (Sakura Finetek Inc.).
4. Leica Cryostat CM 1850 UV (Leica Microsystems).

2.3 LCM Staining

1. Frozen tissue staining: Mayer's hematoxylin solution, eosin Y solution (alcohol-based), and Scott's tap water substitute bluing solution (magnesium sulfate buffered with sodium bicarbonate).
2. Frozen tissue dehydration: 100% ethanol, HPLC-grade. 70% (v/v) and 95% (v/v) ethanol baths: Prepare using LC-MS CHROMASOLV® Water.
3. Final post-dehydration step: Xylene.
4. Precleaned glass microscope slides, 25 × 75 mm.
5. Membrane slide options include:
   (a) Pen-membrane glass slide (Life Technologies, Applied Biosystems).
   (b) Pen-membrane frame slide (Life Technologies, Applied Biosystems).

2.4 LCM Procedure

1. PixCell IIe, Veritas, or ArcturisXT (Life Technologies, Applied Biosystems).
2. CapSure® Macro LCM Caps (Life Technologies, Applied Biosystems).

2.5 LCM-Based Tissue Lysis and Protein Extraction

1. 1 M ammonium bicarbonate (NH_4HCO_3), pH 8.0: Prepare a 100 ml stock solution by dissolving 7.9 g of NH_4HCO_3 in 80 ml of LC-MS CHROMASOLV® water. Mix and adjust pH to 8.0 with formic acid (HCOOH) or ammonium hydroxide (NH_4OH). Adjust the volume to 100 ml with LC-MS CHROMASOLV® water.
2. 12.5 mM ammonium bicarbonate (NH_4HCO_3), pH 8.0: Prepare a 100 ml stock solution by dissolving 1.25 ml of 1 M NH_4HCO_3, pH 8.0 in 98.75 ml of LC-MS CHROMASOLV® water.

3. Prepare a hypotonic lysis buffer (ammonium bicarbonate to methanol: v/v = 80/20, pH ~ 8.0). For convenience, we recommend a 1 ml stock solution prepared by mixing 800 µl of 12.5 mM ammonium bicarbonate with 200 µl of 100% methanol and 2 µl of 0.5 mM TCEP (1 mM final concentration).
4. Methanol, LC-MS grade.
5. 0.5 M Bond-Breaker TCEP Solution, Neutral pH (Thermo Scientific).
6. 1.5 ml siliconized tubes (VWR).

2.6 Protein Concentration Determination

1. Pierce BCA protein assay kit (ThermoFisher Scientific).
2. Corning Costar 96-well microplates.
3. Plate reader capable of measuring at an absorbance of 562 nm.

2.7 1D SDS-PAGE

1. SureLock XCELL mini gel apparatus (Invitrogen Life Technologies).
2. PowerEase 500 power programmable power supply (Invitrogen Life Technologies).
3. NUPAGE 4–12% Bis-Tris gels (Invitrogen Life Technologies).
4. 10× NUPAGE sample reducing agent (Invitrogen Life Technologies).
5. 4× NUPAGE LDS sample buffer (Invitrogen Life Technologies).
6. See Blue Plus 2 pre-stained protein standard (Invitrogen Life Technologies).
7. NUPAGE antioxidant (Invitrogen Life Technologies).
8. 1× NUPAGE MES SDS running buffer (Invitrogen Life Technologies): Add 50 ml of 20× NUPAGE MES SDS running buffer to 950 ml of LC-MS CHROMASOLV® water and mix.
9. Eppendorf Thermomixer R heating block (VWR).
10. Silver stain-compatible gel staining tray.

2.8 Silver Staining the 1D SDS-PAGE Gel

1. SilverQuest Silver Stain Kit (ThermoFisher Scientific):
   (a) *Fixative* (40% ethanol, 10% acetic acid): 40 ml HPLC-grade ethanol, 10 ml HPLC grade acetic acid, 50 ml LC-MS CHROMASOLV® water.
   (b) *30% Ethanol*: 60 ml HPLC-grade ethanol, 140 ml LC-MS CHROMASOLV® water.
   (c) *Sensitizing Solution*: 30 ml HPLC-grade ethanol, 10 ml sensitizer, 60 ml LC-MS CHROMASOLV® water.
   (d) *Staining Solution*: 1 ml stainer, 99 ml LC-MS CHROMASOLV® water.

(e) *Developing Solution*: 10 ml developer, 1 drop developer enhancer, 90 ml LC-MS CHROMASOLV® water.

(f) *Stopper Solution*: 10 ml directly from bottle.

2.9 Gel Band Excision

1. Camera capable of photographing SDS-PAGE gels.
2. ViewOne™ LabLite (Embitec) or other light sources capable of illuminating the gel.
3. Bard-Parker® Disposable Scalpels, Size 11, 371611 (Aspen Surgical).

2.10 In-Gel Digestion

1. Destainer A (from the SilverQuest Silver Stain Kit) (Thermo-Fisher Scientific).
2. Destainer B (from the SilverQuest Silver Stain Kit) (Thermo-Fisher Scientific).
3. 1% Formic Acid: 400 µl LCMS-grade formic acid, 39.6 ml LC-MS CHROMASOLV® water.
4. 25 mM ammonium bicarbonate (NH_4HCO_3), pH 8.0: Prepare a 100 ml stock solution by dissolving 2.5 ml of 1 M NH_4HCO_3, pH 8.0 in 97.5 ml of LC-MS CHROMASOLV® water.
5. Mass spectrometry grade trypsin (20 µg, lyophilized): Add 1 ml of 25 mM NH_4HCO_3, pH 8.0 to 20 µg lyophilized Trypsin. Vortex to mix.
6. 70% acetonitrile/5% formic acid: 28 ml LC-MS-grade acetonitrile, 2 ml LC-MS-grade formic acid, 10 ml LC-MS CHROMASOLV® water.
7. Genie vortex mixer (VWR).
8. Lyophilizer/Speedvac.
9. Shaking 37 °C incubator.
10. Sonicating water bath.

2.11 C18 Peptide Clean-Up (Zip-Tip)

1. ZipTips packed with C18 reversed-phase resin (EMD Millipore).
2. Biohit Proline™ 8-channel or 12-channel Pipette (Sartorius).
3. 10% FA: 4 ml of LC-MS grade formic acid, 36 ml of LC-MS CHROMASOLV® water.
4. 0.1% FA: 400 µl of 10% FA, 39.6 ml of LC-MS CHROMASOLV® water.
5. 50% ACN: 20 ml of LC-MS grade acetonitrile, 20 ml of LC-MS CHROMASOLV® water.
6. 10% TFA stock solution: Add 4 ml of LC-MS grade trifluoroacetic acid to 36 ml of LC-MS CHROMASOLV® water.

7. 0.1% TFA: 400 μl of 10% TFA stock solution, 39.6 ml of LC-MS CHROMASOLV® water.
8. 70% ACN/0.1% TFA: 28 ml of LC-MS grade acetonitrile, 400 μl of 10% TFA stock solution, 11.6 ml of LC-MS CHROMASOLV® water.
9. 0.1% TFA/5% methanol: 400 μl of 10% TFA stock solution, 2 ml of LC-MS-grade methanol, 37.6 ml of LC-MS CHROMASOLV® water.

3 Methods

3.1 Fresh Frozen Tissue Acquisition and Sectioning

In order to obtain optimum molecular results, the tumor sample needs to be handled in a rapid and deliberate fashion. This is critical, since tissue degradation and necrosis may begin following ligation from the blood supply. To minimize ischemic effects, the tissue should be snap frozen in liquid nitrogen and then transferred to a −80 °C freezer. Subsequently, the cryostat-mounting medium is used to embed the tumor tissue, making cryostat cutting easier. Generally, a slice thickness in the range of 8–12 μm is used, and sections are cut serially using a cryostat, from the frozen tissue block embedded in O.C.T.

3.2 Initial Pathologic Evaluation (Prior to LCM)

Prior to LCM analysis, a formal hematoxylin and eosin (H&E) staining procedure with glass cover slip should be performed, on every 10th slide. All the slides require review by a pathologist. The aims are to properly plan the LCM sessions by evaluating the histopathology, with attention to guard against bias in the z-dimension of the tumor tissue plane.

3.3 LCM Staining

Before beginning the staining protocol, the fresh frozen slide must be fully defrosted. This may be accomplished by placing the slide in the palm of your glove. The staining protocol may begin as soon as condensate forms on the entire slide. To aid with tissue capture and visualization, the times below are recommended for membrane and glass slides (*see* **Note 1**).

Step	Solution	Comment	Time (membrane slide)	Time (glass slide)
1	70% ethanol	Fix tissue section to slide	15 s	30 s
2	LC-MS CHROMASOLV® Water	Remove OCT, rehydrate tissue	30 s	30 s
3	Hematoxylin	Stain nuclei	45 s	30 s

(continued)

4	LC-MS CHROMASOLV® Water	Remove excess hematoxylin	15 s	30 s
5	Bluing solution	Change hematoxylin hue	15 s	30 s
6	70% ethanol	Start dehydration	15 s	30 s
7	Eosin	Stain cytoplasm (1–2 quick dips)	1–2 s	2 s
8	95% ethanol	Dehydration	30 s	1 min
9	95% ethanol	Dehydration	30 s	1 min
10	100% ethanol	Dehydration	30 s	2 min
11	100% ethanol	Dehydration	30 s	2 min
12	Xylene	Ethanol removal	3 min	3 min

3.4 LCM Procedure

Once the slides are air-dried, LCM analysis may begin. Dissections approaching 100% purity are typically achieved for laser-based systems. Proper microscopic visualization during the dissection process is critical, and H&E staining provides for this while not diminishing protein recovery. Depending on the type of tissue under study, we have found an absolute minimum of 5000–10,000 cells are required to produce MS results with an acceptable number of protein identifications as well as providing an acceptable degree of protein class diversity (*see* **Note 2**).

3.5 LCM-Based Tissue Lysis and Protein Extraction

The LCM tissue lysis and protein extraction protocol was adapted and modified from work published by our laboratory [17] and previous researchers [18].

1. Carefully remove the LCM polymer membrane by peeling it off the CAP and then place it in a 1.5 ml siliconized tube (conical bottom).
2. Add 50 μl of hypotonic lysis buffer.
3. Incubate on dry ice for 30 min.
4. Thaw the sample in ice-cold water for 10 min.
5. Incubate the sample in a water bath for 2 h at 70 °C.
6. Cool the sample on ice for 20 min.
7. Adjust the buffer from 12.5 to 50 mM by adding 1.65 μl of 1 M ammonium bicarbonate.
8. Microcentrifuge the LCM tissue homogenate at 4 °C for 5 min at 10,000 × *g*.
9. Collect the supernatant and pipette into a fresh 1.5 ml siliconized tube (conical bottom).

3.6 Protein Concentration Determination

Perform the BCA assay as per manufacturer's instructions: Pierce BCA Protein Assay Kit.

3.7 1D SDS-PAGE Gel

1. Assemble the SureLock XCELL mini gel apparatus with a 4–12% Bis-Tris gel and 1× MES SDS running buffer as per manufacturer's instructions.
2. Transfer 1–5 μg of the LCM tissue homogenate to a 0.5 ml Eppendorf tube.
3. Add LC-MS CHROMASOLV® water, 10× NUPAGE reducing agent, and 4× NUPAGE sample buffer according to the following manufacturer's recommendations:

Reagent	Sample
1–5 μg LCM Tissue Homogenate	X μl
LC-MS CHROMASOLV® Water	to 13 μl
10× NUPAGE Reducing Agent	2 μl
4× NUPAGE LDS Sample Buffer	5 μl
Total Volume	**20 μl**

4. Heat the tissue homogenate for 10 min at 70 °C.
5. Load the tissue homogenate in the well of a 4–12% Bis-Tris gel.
6. Load 15–20 μl of See Blue Plus 2 pre-stained protein standard in an adjacent well.
7. Perform gel electrophoresis at 125 V.
8. Remove the gel from the cassette, place the gel in a silver stain-compatible gel staining tray containing enough LC-MS CHROMASOLV® water to cover the gel (approximately 200 ml), and incubate on a platform rocker for 5 min.
9. Stain the gel using SilverQuest Silver-stain kit (*see* **Note 3**).

3.8 Silver Staining the 1D SDS-PAGE Gel

The 1D SDS-PAGE gel is stained using SilverQuest Silver-Stain Kit as per manufacturer's instructions as follows:

1. Decant the LC-MS CHROMASOLV® water.
2. Fix the gel in 100 ml of Fixative. Incubate for 1 h on a platform rocker at room temperature.
3. Decant Fixative and wash the gel in 100 ml of 30% ethanol. Incubate for 10 min on a platform rocker at room temperature.
4. Decant 30% ethanol and incubate the gel in 100 ml of Sensitizing Solution. Incubate for 10 min on a platform rocker at room temperature.

5. Decant Sensitizing Solution and wash the gel in 100 ml of 30% ethanol. Incubate 10 min on a platform rocker at room temperature.
6. Decant 30% ethanol and wash the gel in 100 ml of LC-MS CHROMASOLV® water. Incubate for 10 min on a platform rocker at room temperature.
7. Decant water and incubate the gel in 100 ml of Staining Solution. Incubate for 15 min on a platform rocker at room temperature.
8. Decant Staining Solution. Wash the gel in 100 ml of LC-MS CHROMASOLV® water for 20–60 s on a platform rocker at room temperature.
9. Decant water and incubate the gel in 100 ml of Developing Solution until bands appear and the desired band intensity is reached.
10. Add 10 ml of Stopper solution and incubate the gel for 15 min at room temperature to stop the staining reaction.
11. Decant the solution and wash the gel in 200 ml of LC-MS CHROMASOLV® water. Incubate for 10 min on a platform rocker at room temperature.
12. Store the gel in LC-MS CHROMASOLV® water at 4 °C if gel bands are to be excised at a later date.

3.9 Gel Band Excision

1. Photograph the gel.
2. Generate a gel excision scheme using the photograph of the gel (Fig. 2) (*see* **Note 4**).
3. Excise the gel bands of interest using a scalpel (Fig. 3) and place each gel band in a separately labeled 1.5 ml eppendorf tube.
4. Using a scalpel, dice each gel band into small pieces (*see* **Note 5**).
5. Store at −80 °C if the in-gel digestion is to be performed at a later date.

3.10 In-Gel Digestion

In-gel digestion is performed over the course of 2 days. Day one entails de-staining and protein digestion with trypsin. Peptides are extracted from the gel on day two.

1. Wash the gel pieces for 5 min by adding 200 µl of LC-MS CHROMASOLV® water to each eppendorf tube.
2. Centrifuge for 1 min at 10,000 × *g* to pellet the gel pieces.
3. Destain the gel pieces by adding 50 µl/eppendorf tube of Destainer A and 50 µl/eppendorf tube of Destainer B (*see* **Note 6**).
4. Incubate for 15 min @ room temperature on a vortex mixer.

5. Centrifuge for 1 min at 10,000 × *g* to pellet the gel pieces.
6. Aspirate and discard the destaining solution from each eppendorf tube.
7. Wash the gel pieces by adding 200 µl of LC-MS CHROMASOLV® water to each eppendorf tube. Incubate on a vortex mixer for 5 min.
8. Centrifuge for 1 min at 10,000 × *g* to pellet the gel pieces.
9. Aspirate and discard the water wash from each eppendorf tube.
10. Repeat **steps 7–9**.
11. Add 200 µl of 1% formic acid to each eppendorf tube. Incubate on a vortex mixer for 5 min.
12. Centrifuge for 1 min at 10,000 × *g* to pellet the gel pieces.
13. Aspirate and discard the formic acid.
14. Lyophilize the gel pieces to dryness.
15. Prepare a trypsin solution (*see* **Notes 7** and **8**): Add 1 ml of 25 mM NH_4HCO_3, pH 8.4 to 20 µg of sequencing grade modified porcine trypsin and Add just enough trypsin solution to each eppendorf tube to cover the gel pieces.
16. Incubate each eppendorf tube on ice for 45 min.
17. Remove excess trypsin from each eppendorf tube.
18. Cover the gel pieces with 25 mM NH_4HCO_3, pH 8.0 (*see* **Note 9**).
19. Incubate overnight at 37 °C.
20. Aspirate supernatants (25 mM NH_4HCO_3) to fresh 0.5 ml eppendorf tubes.
21. Extract peptides three times each with 70% acetonitrile/5% formic acid as follows:
    (a) Add 50 µl of 70% acetonitrile/5% formic acid to each eppendorf tube containing gel pieces.
    (b) Sonicate at room temperature for 10 min in a sonicating water bath.
    (c) Aspirate the 70% acetonitrile/5% formic acid and add it to the 0.5 ml eppendorf tube containing the supernatants.
    (d) Repeat **steps a–c** twice.
22. Lyophilize to dryness and store at −80 °C until C18 peptide clean-up.

3.11 C18 Peptide Clean-Up (Zip-Tip)

Our laboratory has developed a method to apply the Zip-Tip protocol to eight or twelve channel pipettes. This allows for clean-up of up to twelve samples simultaneously. A single-channel pipette and multichannel pipette Zip-Tip protocol is presented below.

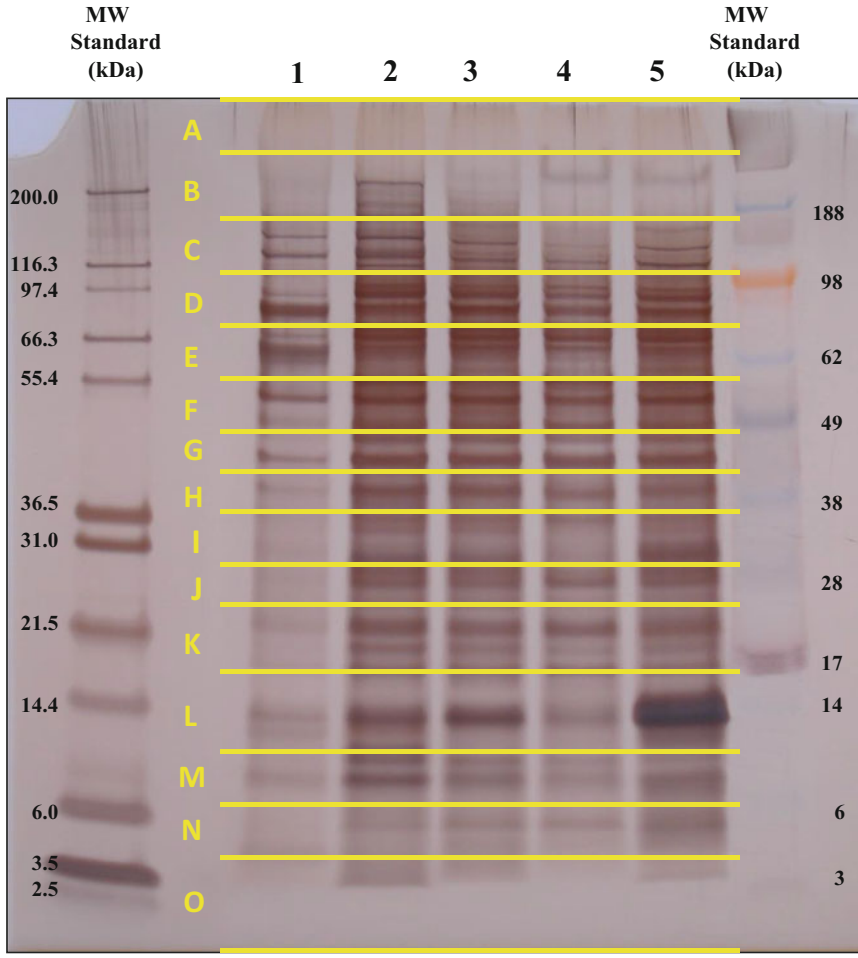

Fig. 2 Example of a 1D SDS-PAGE gel excision scheme

3.11.1 Single-Channel Pipette Protocol (for <8 Samples) (See ***Notes 10*** *and* ***11****)*

1. Resuspend lyophilized peptides in 25 µl of 0.1% Formic Acid.
2. Add 25 µl of 70% ACN/ 0.1% TFA to a fresh eppendorf tube. Set aside.
3. Wet zip-tip once with 25 µl of 50% ACN (Draw 25 µl through the Zip-Tip using a pipette and expel the liquid into a waste container).
4. Pre-equilibrate the tip twice with 25 µl of 0.1% TFA (Draw 25 µl through the Zip-Tip using a pipette and expel the liquid into a waste container; repeat).
5. Bind resuspended peptides to the C18 resin (Draw resuspended peptides through the Zip-Tip using a pipette; pipette up and down 20–30 times).

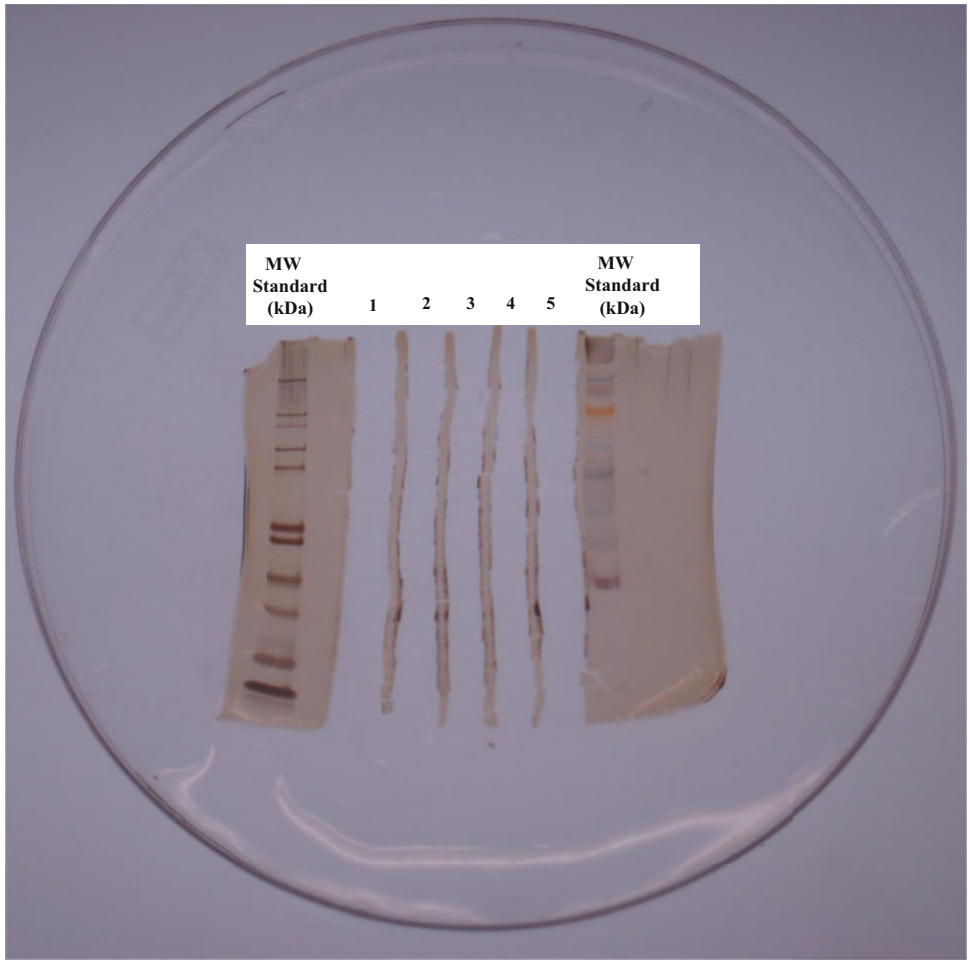

Fig. 3 1D SDS-PAGE gel with excised gel bands

6. Wash the peptides three times with 25 μl of 0.1% TFA/5% methanol (Draw 25 μl through the Zip-Tip using a pipette and expel the liquid into a waste container; repeat twice).
7. Elute the peptides from the Zip-Tip C18 resin into the fresh tube containing 70% ACN/ 0.1% TFA by slowly pipetting up and down 20–30 times.
8. Lyophilize the peptides to dryness and store at −80 °C until LC-MS analysis (*see* **Note 12**).

3.11.2 Multi-Channel Pipette Method (for ≥8 Samples) (See ***Notes 11*** *and* ***13****)*

1. Resuspend the lyophilized peptides in 25 μl of 0.1% Formic Acid.
2. Set up the following:
   (a) Three separate sample reservoirs labeled and containing:

- 50% ACN
- 0.1% TFA
- 0.1% TFA/5% MeOH

(b) A 96-well plate containing two rows of 0.5 ml eppendorf tubes:

Row 1: 8–12 sample tubes (depending upon the number of samples to be processed) containing the peptides resuspended in 0.1% formic acid.
Row 12: 8–12 fresh 0.5 ml eppendorf tubes (depending upon the number of samples to be processed) each containing 25 μl of 70% ACN/ 0.1% TFA.

3. C18 Zip-Tip:

(a) Using an 8-channel or 12-channel pipette, wet zip-tips once with 25 μl of 50% ACN (Draw 25 μl through the Zip-Tips using a multichannel pipette and expel the liquid into a waste container).

(b) Pre-equilibrate the tips twice with 25 μl of 0.1% TFA (Draw 25 μl through the Zip-Tips using a multi-channel pipette and expel the liquid into a waste container; repeat).

(c) Bind the resuspended peptides in Row 1 of the 96-well plate to the Zip Tip C18 resin (Draw resuspended peptides through the Zip-Tips using a multichannel pipette; pipette up and down 20–30 times).

(d) Wash the peptides three times with 25 μl of 0.1% TFA/5% methanol (Draw 25 μl through the Zip-Tips using a multichannel pipette and expel the liquid into a waste container; repeat twice).

(e) Elute the peptides from the Zip-Tips into the fresh tubes in Row 12 of the 96-well plate containing 70% ACN/0.1% TFA by slowly pipetting up and down 20–30 times.

(f) Lyophilize the peptides to dryness and store at −80 °C until LC-MS analysis (*see* **Note 12**).

4 Notes

1. For each step in the staining protocol, a different solution bath is recommended. Through experience, this procedure has been found to make a significant difference to subsequent analyses. Additionally, when the glass slides are used for LCM, the time for dehydration (**steps 8–11**) may need to be increased up to 1 min (occasionally up to 3 min) for each ethanol bath. The

increased time may improve the pickup of captured cells from the glass slide onto the LCM Cap. Finally, enhanced dehydration is usually not required for membrane slides.

2. We have found that tissues with a compact cellular density provide greater protein yields and thus usually require a smaller quantity of cells. However, when encountering a new tumor tissue type, a few preliminary experiments are recommended to determine the general estimate of protein yield.
3. The SilverQuest Silver Stain Kit is MS compatible. This stain does not interfere with further down-stream sample processing steps, nor does it interfere with LC-MS applications.
4. The gel excision scheme is generated according to the photograph of your gel and the desired number of bands to be excised.
5. The gel pieces should be small, but not small enough to be aspirated into a 100–200 μl pipette tip.
6. Use Destainer A & Destainer B from the SilverQuest Silver Stain Kit.
7. The lyophilized gel pieces will rapidly rehydrate. Additional trypsin solution may need to be added to some tubes. Remember, add just enough trypsin solution to cover the gel pieces. Do not drown the gel pieces in trypsin solution.
8. Excised gel bands vary in size; therefore, variable amounts of trypsin solution may be warranted for each eppendorf tube.
9. Keep in mind that the samples will be digested with trypsin overnight at 37 °C. Add a sufficient amount of 25 mM NH_4HCO_3, pH 8.0 to withstand this overnight incubation.
10. This method utilizes a single-channel pipette and processes one sample at a time. This procedure is time-consuming.
11. Be careful not to draw bubbles into the Zip-Tip as this may reduce the efficiency of peptide binding to the C18 resin. Do not allow the Zip-Tip C18 resin to dry.
12. For LC-MS analysis, resuspend the lyophilized peptides in 10–12 μl of 0.1% formic acid or 10–12 μl of 0.1% TFA.
13. This method utilizes an 8-channel pipette or a 12-channel pipette and processes multiple samples at one time. Our laboratory has found this to be the preferred protocol. It is less laborious. We have found this modification to be efficient and second only to automation.

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Chapter 5

Next-Generation Sequencing Analysis of Laser-Microdissected Formalin-Fixed and Paraffin-Embedded (FFPE) Tissue Specimens

Lavinia Mägel, Stephan Bartels, and Ulrich Lehmann

Abstract

In recent years, next-generation sequencing (NGS) became widely used in molecular pathology. Comprehensive mutational profiling improved diagnosis and prognosis, as well as the identification of therapeutically relevant genetic alterations. However, the vast majority of studies analyzing tissue samples use DNA extracted from bulk tissue or only manually microdissected specimens. Laser-assisted microdissection offers the possibility of isolating morphologically defined small tissue compartments (like individual glands) or even of single cells for further molecular analysis. Even formalin-fixed paraffin-embedded (FFPE) tissue specimens can be used for laser-assisted microdissection. Combining these two innovative powerful methodological approaches provides invaluable insights into the genetic profile of any cell type and tissue compartment of interest, contributing to a better understanding of fundamental biological processes and disease-specific mechanisms.

In this chapter, a detailed protocol is provided for microdissection of human mammary adenomyoepithelioma tissue specimens and subsequent targeted resequencing of a panel of cancer-related genes using IonTorrent/PGM technology.

Key words Laser-Microdissection, Next-generation sequencing, Mutation detection, Cell type-specific, Formalin-fixed and paraffin-embedded (FFPE)

1 Introduction

Next-generation sequencing and laser-assisted microdissection are both "disruptive" methodologies which brought unthinkable opportunities to the analysis of tissue samples in life science and medicine. The technical revolution in DNA sequencing witnessed during the last years enables now the routine analysis of dozens or even hundreds of genes for diagnosis, prognosis or prediction within 5–10 working days even in small-scale laboratories with moderate sample throughput [1, 2]. Laser microdissection made possible the molecular analysis of morphologically defined and immunohistochemically characterized tissue compartments or

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even single cells isolated from solid tissue samples [3–5]. The combination of both methodologies will increase our molecular understanding of physiological processes and diseases even further.

Starting in the nineties several microdissection devices using a laser beam as a dissection tool have been developed [6, 7]. The MMI CellCut plus laser-microdissection system from Molecular Machines & Industries (Eching; Germany) followed the pioneers from P.A.L.M. and Acturus and is a robust and reliable tool for contamination-free isolation of single cells or small groups of cells. It allows sample inspection at high magnification at high quality and cells of interest are easily dissected due to solid state laser technology creating a very focused laser beam and a user-friendly operation software (Software Cellcut 2.0). This technique is especially useful for dissection of single cells like megakaryocytes or lung epithelial cells [4, 5]. Combination of this laser-microdissection system and next-generation sequencing allows comprehensive mutation profiling of specific, morphologically defined cell types or tissue compartments.

2 Materials

Manufacturers or distributors are specified only if reagents or laboratory equipment might be important for the outcome or if a source might be difficult to identify.

2.1 Tissue Fixation

1. Tissue dissection tools.
2. Tissue cassettes.
3. 4% Buffered formalin.
4. 100% and 70% Ethanol.
5. Xylene.
6. Paraffin wax.

2.2 Slide Preparation and Laser-Assisted Microdissection

1. MMI-Membrane Slides, nuclease and human nucleic acid free 1.4 μm thick PET-membrane (Molecular Machines & Industries, Eching; Germany).
2. MMI-Adhesive-Isolation Caps 500 μL, diffuse (Molecular Machines & Industries, Eching; Germany).
3. Microtome (standard version for histological laboratories).
4. Glass slides (standard version for histological laboratories).
5. MMI CellCut Plus laser-microdissection system (Molecular Machines & Industries, Eching; Germany).
6. Microscope Olympus IX71.

2.3 Immunohistochemistry

1. p63: monoclonal mouse anti-human antibody, clone 4A4, Biocare Medical, Concord, CA, USA.
2. Horseradish peroxidase (Zytomed-Systems, Berlin, Germany).

2.4 DNA Isolation

1. 20 mg/mL Proteinase K.
2. Proteinase K buffer: 50 mM Tris–HCl pH 8.1, 1 mM EDTA, and 0.5% Tween 20.
3. 3 M Sodium acetate, pH 7.
4. DextranT500 (20%w/v solution).
5. Thermoshaker.
6. Refrigerated tabletop centrifuge.
7. Ethanol.
8. TE buffer: 10 mM Tris–HCl pH 8.1, 1 mM EDTA.
9. 0.5 mL DNA/RNA LoBind microcentrifuge tubes (e.g., from Eppendorf, Hamburg, Germany, *see* **Note 1**).

2.5 Next-Generation Sequencing

1. Next-generation sequencing system (e.g., IonTorrent™ PGM, *see* **Note 2**).
2. DNA Library preparation kit for Next-generation sequencing (e.g., Ion AmpliSeq™ Library Kit 2.0).
3. Customized or commercially available primer Panel (e.g., Ion AmpliSeq™ Colon and Lung Research Panel v2).

3 Methods

The following steps describe FFPE-based tissue fixation and subsequent next-generation sequencing analysis from laser-microdissected specimens:

Tissue fixation.
Slide preparation and laser-assisted microdissection.
DNA isolation.
Next-generation sequencing.
Data interpretation.

3.1 Tissue Fixation

1. Prepare tissue (organ specimens, biopsies, etc.) with dissecting tools as required (scalpel, scissors, tweezers, etc.). Section the sample into thin slices and place into a tissue cassette, taking care not to fill up more than approximately two third of the cassette's volume. The maximum thickness should not exceed 5 mm (formalin penetration is about 1 mm/h).
2. Transfer cassette into formalin for 12–20 h. The ratio formalin/tissue should ideally be 20:1 or higher. Lower ratios might result in inadequate fixation and degrading of nucleic acids.

3. Transfer cassette into graded ethanol to dehydrate (70% for 1 h, 90% for 45 min, 100% for 45 min, 100% for 1 h [no. 1], 100% for 1 h [no. 2], 100% for 1 h [no. 3]; 40 °C).
4. Transfer cassette into xylene (for 45 min [no. 1], for 45 min [no. 2], for 1 h) (*see* **Note 3**).
5. Transfer cassette into paraffin wax (for 30 min [no. 1], for 30 min [no. 2], for 30 min [no. 3]; 62 °C).
6. Embed sample into a paraffin block.

3.2 Slide Preparation, Immunohistochemistry, and Laser-Assisted Microdissection

Work under nuclease-free conditions. Here, we take as an example the laser-assisted microdissection of human mammary adenomyoepithelioma tissue specimens. Thereby, we collect separately basal myoepithelioma and luminal cells.

1. Cut 5–10 μm thick serial sections with a fresh blade from the paraffin block and float them out on a warm water bath (approx. 45 °C) (*see* **Note 4**).
2. Mount sections on nuclease-free membrane slides. You can store the prepared slides until usage for a couple of days at 4 °C.
3. Perform immunohistochemistry to visualize basal myoepithelial cells by staining p63 with antigen retrieval (citrate buffer, pH 6, 98 °C) and visualization by horseradish peroxidase followed by DAB incubation.
4. Use haematoxylin for counter-staining.
5. In order to stabilize the very thin quite fragile membrane slides, place a regular glass slide under each membrane slide.
6. Use the MMI CellCut Plus system to collect basal myoepithelial cells and luminal cells of tissue samples of patients with adenomyoepithelioma in different tubes with an adhesive lid (*see* Fig. 1). Set the power and magnification appropriately to the size of the target structures, the target tissue and the thickness of the sections (*see* **Note 5**). In our experience a 100× magnification works best for most tissues.
7. Collect about 3000–5000 cells per compartment (*see* **Note 6**).

3.3 DNA-Isolation

1. Add 80 μL Proteinase K buffer and 10 μL Proteinase K into each of the adhesive-cap centrifuge tubes with the microdissected tissue on the adhesive-cap.
2. Lyse the samples overnight in a Thermoshaker at 55 °C with 550 rpm (*see* **Note 7**).
3. Add 10 μL 3 M sodium acetate (with 100 μg/mL Dextran).
4. Add 2.5 volume of 100% Ethanol (250 μL).
5. Precipitate the DNA overnight at −20 °C.
6. Centrifuge at 12,000 × *g* at 4 °C for 20 min.

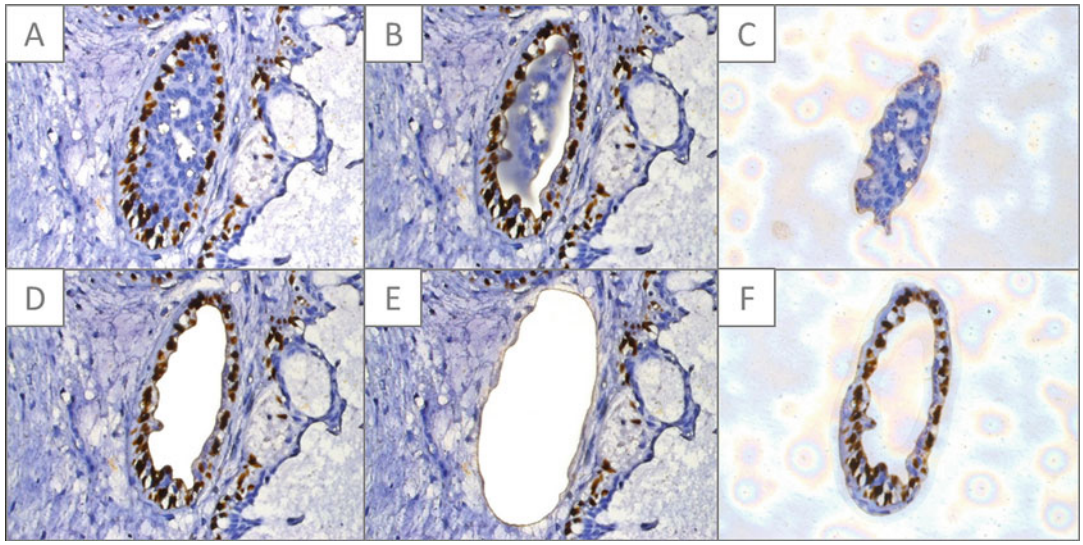

Fig. 1 Laser-assisted microdissection. Myoepithelial cells of mammary glands were stained for p63 (horseradish peroxidase, brown) and nuclei were counterstained with hemalaun (**a**). First, luminal cells of mammary glands were dissected (**b**) and collected by using adhesive isolation caps (**c**). Subsequently myoepithelial cells were cut out (**d**, **e**) and collected in another cap (**f**). Original magnification: ×400

7. Discard the supernatant and wash the pellet with 100 μL of 70% ethanol.
8. Centrifuge at 12,000 × *g* at 4 °C for 5 min.
9. Discard the supernatant, air-dry the pellet for 5 min, and elute in 25 μL (or less) of TE buffer.

3.4 Next-Generation Sequencing

Detailed protocol handbooks describing and explaining the laboratory workflow of the various commercially available next-generation sequencing library preparation methods and kits can be obtained from the respective manufacturer. As already mentioned we use the Ion Torrent/PGM platform and follow the protocols provided by the manufacturer without any deviation (*see* ref. 8). In a previous publication we could show that hybrid capture based library preparation is possible with DNA extracted from FFPE tissue specimens [9], but for laser-microdissected specimens we would recommend amplicon-based library preparation, which results in higher library concentrations. In our hands, Illumina® amplicon-based library preparation kits did not yield optimal results with low amounts of DNA input (resulted in high number of sample drop outs). Small IonTorrent™ panels (containing less than 100 amplicons) or small customized panels together with IonTorrent™ amplicon-based library kits worked well in our lab, even with very low amounts of DNA input. We have no hands-on experience with Qiagen library preparation technology for the analysis of laser-microdissected specimens.

Working with limited amounts of nucleic acids obtained by laser-microdissection a strict separation of pre-PCR area and post-PCR area is absolutely mandatory (*see* **Note 8**). This is even more important if patient samples are processed for molecular pathological diagnosis.

3.5 Data Evaluation

In this chapter, microdissection of human mammary adenomyoepithelioma is taken as an example for lesions where different histological structures can be identified and analyzed. With the help of next-generation sequencing the mutation profile of the laser-microdissected compartments can be compared. This can answer the question in which specific tissue the lesion originates. Or, in other circumstances, characterization of two independent tumors in one patient is possible.

A bioinformatics pipeline for data evaluation is indispensable for the distinction between single-nucleotide variants (SNPs) and pathogenic mutations [8]. For this purpose a number of huge openly accessible databases exist (1000 Genomes, ESP6500, dbSNP, ClinVar, ExAC). Furthermore, different in silico prediction tools can help to evaluate the functional relevance of unknown variants (e.g., MutationTaster SIFT, PolyPhen2). We strongly recommend checking all unusual rare sequence variants manually using the IGV browser.

4 Notes

1. The amount of extracted DNA is often very limited; depending on cell count (typically 3000–5000 cells per tissue specimen) up to 1 ng/μL (25 ng in total) is possible. To reduce further DNA loss, LoBind microcentrifuge tubes are recommended for the entire workflow. These tubes have an optimized surface which lowers the binding of nucleic acids.
2. In our experience, very limited amounts of possible DNA input exclude downstream Illumina library preparation. The IonTorrent™ Ampliseq™ library preparation chemistry works reliably with a total DNA input down to 2 ng.
3. In our institution **steps 2–4** are routinely performed in an automatic tissue processing device.
4. Regular meticulous cleaning is absolutely essential. In our institution the water bath is always prepared fresh immediately before use with nuclease-free water.
5. The optimal settings of laser energy level and focus depend largely on the tissue that has to be cut. It is always advisable to test the settings on a marginal area of the section before moving on to the region of interest within the tissue section.

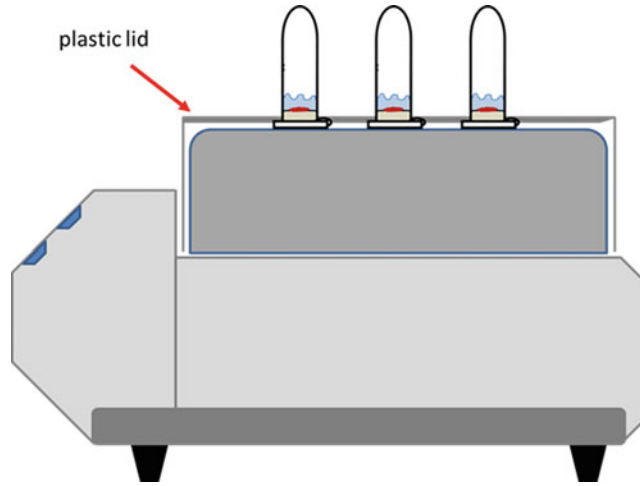

Fig. 2 Schematic picture of a thermoshaker with adhesive-cap centrifuge tubes placed upside-down

Remember that a significant reduction of the cutting speed is also an efficient way to increase the penetration depth of the laser beam. In general, very small-target structures should be cut out slowly with reduced laser energy. For larger structures the energy and the velocity can be increased.

6. Assuming that a human diploid cell contains approx. 6.5 pg genomic DNA theoretically this results in 20–32 ng of genomic DNA. However, due to the sectioning the majority of nuclei are not complete any more. And further losses during the subsequent steps are unavoidable. Therefore, one can expect 5–10 ng of genomic DNA.
7. For the lysis of the microdissected tissue into the adhesive-cap centrifuge tubes overnight we recommend placing the centrifuge tubes upside-down into the Thermoshaker (*see* Fig. 2). For an optimal distribution of the temperature, the tubes can be covered with aluminum foil.
8. Due to the risk of contamination, a strict physical separation of reaction products from all sample preparation has to be accomplished. To this end, we maintain a laboratory (pre-PCR-area), where sectioning and laser-assisted microdissection and PCR setup is performed. Everything in this laboratory (including lab coats, pipettes, notepads, etc.) is dedicated exclusively to this room and strictly separated from the post-PCR-area. Workplaces and plastic labware are regularly cleaned using a 3% hypochlorite solution. Under no circumstances should amplified samples or equipment from this laboratory be brought back to the pre-PCR laboratory. This requires special education of a dedicated staff and regular briefings about the organization of the laboratories and the guidelines for sample processing.

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Chapter 6

Adaptation of Laser Microdissection Technique to Nanostring RNA Analysis in the Study of a Spontaneous Metastatic Mammary Carcinoma Mouse Model

Nadia P. Castro and Yelena G. Golubeva

Abstract

The mouse model characterized by spontaneous lung metastasis from JygMC (A) cells closely resembles the human triple negative breast cancer (TNBC) subtype. The primary tumors morphologically present both epithelial and spindle-like cells, but metastases in lung parenchyma display only adenocarcinoma properties. In the study of molecular signatures, laser capture microdissection (LCM) on frozen tissue sections was used to separate the following regions of interest: the epithelial–mesenchymal transition (EMT), mesenchymal–epithelial transition (MET), carcinoma, lung metastases, normal mammary gland and normal lung parenchyma. NanoString was selected for the study of molecular signatures in LCM targets as a reliable downstream gene expression platform allowing analysis of tissue lysates without RNA extraction and amplification. This chapter provides detailed protocols for the collection of tissue, LCM sample preparation and dissection, production of lysates, extraction, and quality control of RNA for NanoString analysis, as well as the methodology of Nanostring gene expression profiling experiment.

Key words NanoString analysis, TNBC mouse model, RNA, Mammary gland, Laser capture microdissection

1 Introduction

LCM is a research tool used for molecular profiling of defined cell populations which can be cleanly separated from surrounding cells. LCM facilitated the discovery of biomarkers and genes responsible in the disease onset and progression [1–10]. The molecular analysis of complex tissues is not reliable for finding subtle changes in the molecular signature and the correlation of cellular molecular signatures with specific cell populations [9]. Frozen samples are considered to be the base for accurate molecular data, with established protocols for target collection, LCM slide, and lysis preparation [4, 6, 7, 11]. However, these procedures are not directly applicable to the input sample requirements of downstream targets assessment by NanoString nCounter gene expression analysis, which we used

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to study a clinically relevant mouse model. This model phenotypically and at the gene-expression level, resembles the human TNBC molecular subtype which metastasizes spontaneously to the lungs [12]. Combining LCM with NanoString technology we made it possible to focus on highly defined histological areas to clarify discrete molecular changes in gene expression at a greater level of resolution. LCM samples usually contain nanograms of total RNA and require RNA extraction and amplification steps to satisfy input requirements in micro array gene profiling platform. To consider the issue of RNA loss during extraction and possible bias during RNA amplification, we selected a hybridization-based gene profiling platform, Nanostring, where the lysates of LCM targets are used as a direct input to the downstream application. NanoString is a highly advantageous platform for the analysis of LCM samples, as it does not require RNA extraction and amplification, and uses feasible input amounts of RNA; 100 ng of total RNA lysed in 4 μl of RNA extraction buffer [13, 14]. When microarray analysis of RNA extracted from whole tumors is compared with NanoString analysis of LCM lysates from defined cell populations collected within those tumors, it shows that Nanostring is a reliable downstream platform for analyzing gene expression in LCM samples [12, 15]. In the present chapter, we describe the adaptation of LCM methodology for collecting samples suitable for NanoString analysis, which allows high-quality RNA lysates to be obtained from homogenous cell populations, as opposed to heterogeneous whole tissue samples. Our adaptation of LCM sample preparation and workflow to the requirements of the NanoString platform allowed acquiring samples with high RNA quality for sensitive detection of genes of interest during the study of spontaneous metastatic carcinoma mouse model [12, 15]. This workflow is suitable for the study of any mouse model warranting the use of LCM technology for the molecular characterization of specific cell populations.

2 Materials

2.1 Tissue Sample Collection for LCM

1. Necropsy protocol (*see* **Note 1**).
2. Necropsy station with RNase-free forceps, scissors, scalpels, razor blades (*see* **Note 2**).
3. RNase AWAY™.
4. Nuclease-free water.
5. Kimwipes.
6. Euthanasia chamber.
7. CO_2.
8. Tissue-Tek OCT Compound (OCT) (Sakura Finetek USA, Inc., Torrance, CA, USA).

9. Cryomolds.
10. Adhesive Cryolabels (*see* **Note 3**).
11. 2-methylbutane.
12. Dry ice.
13. Styrofoam box.
14. Ziploc bag.

2.2 Cryosectioning of OCT-Embedded Samples for LCM Slide Preparation and RNA Quality Control in OCT Blocks

1. **Items 3–5**, **12** and **13** from Subheading 2.1.
2. Cryostat.
3. OCT tissue blocks.
4. Razor blades.
5. 100% ethanol.
6. Forceps.
7. 1.5 ml nuclease-free micro-centrifuge tube for high G-force.
8. RNase-free glass histology slides.
9. Metal frame PET membrane slides (frame membrane slide) (MMI Molecular Machines & Industries, Glattbrugg, Switzerland).
10. MMI SupportSlide (SupportSlide) (MMI Molecular Machines & Industries Glattbrugg, Switzerland).
11. Adhesive slide labels.
12. Ultraviolet light chamber at 352 nm.
13. RA Lamb Five-Slide Mailer (Five-Slide Mailer).

2.3 Determination of RNA Quality in Tissue Samples Collected for LCM

1. AllPrep® DNA/RNA Micro Kit (Qiagen GmbH, Hilden, Germany).
2. Vortexer.
3. NanoDrop™ Spectrophotometer (NanoDrop).
4. Agilent 2001 Bioanalyzer.
5. Agilent RNA 6000 Pico Chip (Pico Chip) (Agilent Technologies, Santa Clara, CA).
6. Agilent Bioanalyzer 2001 Expert software (Agilent Technologies, Santa Clara, CA).

2.4 Digital Images of H&E Slides for LCM Pathology Annotation Reference

1. **Item 5** from Subheading 2.2.
2. Hematoxylin.
3. Eosin Y.
4. Bluing reagent.
5. Xylene.
6. Aperio Scan Scope®XT scanner.
7. Image Scope™ software.

2.5 Staining of Metal Frame Membrane Slides for Laser Dissection

1. **Items 4**, **5**, **12** and **13** from Subheading 2.1; **item 5** from Subheading 2.2; **item 5** from Subheading 2.4.
2. 50 ml conical polypropylene tubes.
3. ProtectRNA™RNase inhibitor (ProtectRNA) (Sigma, Saint Louis, MO, USA).
4. Glacial acetic acid.
5. MethylGreen (MG).
6. Cresyl violet acetate: 1% solution in 100% ethanol aged for 6 months [11].
7. Eosin Y.
8. Desiccator.
9. Desiccant.

2.6 Laser Microdissection and Pilot Study for the Determination of RNA Quality and Content in LCM Targets

1. **Item 9** from Subheading 2.2.
2. MMI Cell Cut Plus Laser Microdissection System (MMI Cell Cut Plus) (MMI Molecular Machines & Industries, Glattbrugg, Switzerland).
3. Computer with wide monitor for displaying LCM reference images from Aperio database.
4. MMI IsolationCap® (0.5 ml and 0.2 ml) (IsolationCap).
5. Metal frame slides with mounted sections.
6. Conventional dissecting microscope.

2.7 Preparation of LCM Lysates for NanoString Analysis

1. **Item 5** from Subheading 2.1; **items 3** and **5** from Subheading 2.2; **item 6** from Subheading 2.6.
2. RNase-free Inox #5 forceps (Roboz Surgical Instrument, Co., Dumont, Switzerland).
3. Nuclease-free PCR tubes.
4. Buffer RLT (Qiagen GmbH, Hilden, Germany).
5. Centrifuge.
6. Wet ice.
7. RNaseOut™ RNase inhibitor (RNaseOut) (Invitrogen, Carlsbad, CA, USA).
8. RNase-free barrier long tips for 20-PT pipette.

2.8 NanoString Analysis of LCM Samples

1. NanoString technology consumables (NanoString Technologies, Seattle, WA, USA).
2. nCounter Custom Gene Expression Assay (Reporter Codeset and Capture Probeset).
3. Total RNA lysates of LCM samples in buffer RLT.

4. NanoString nSolver Analysis Software version 1.1 (NanoString Technologies, Seattle, WA, USA).
5. NanoString Analysis protocol.

3 Methods

3.1 Tissue Sample Collection for LCM

For the study of a spontaneous metastatic mammary carcinoma mouse model we used female Balb/c athymic nude mice that were 8 weeks of age (National Cancer Institute), which were injected bilaterally into the fourth mammary fat pad with JygMC (A)-GFP/Luc cells under analgesia by isoflurane/O_2 (to effect) [12]. We adopted the following approach for the animal sample collection (*see* **Note 4**)

1. Select eight tumor-bearing mice for the collection of two primary mammary carcinoma masses and two lung metastases from each mouse. Select three untreated animals of the same age for the collection of normal mammary gland and lung parenchyma from each animal (*see* **Note 5**).
2. Conduct necropsy procedures under conditions approved by your institute and ACUC study protocol (*see* **Note 6**).
3. Following RNase-free conditions, prepare the following items: necropsy station, labeled cryomolds, slurry of dry ice and 2-methylbutane in a covered Styrofoam box or ice bucket, a Styrofoam box with dry ice (Fig. 1).
4. Perform euthanasia by exposure to compressed CO_2 gas at a fill rate of 20% of the chamber volume per minute, with one animal per chamber in its home cage. After 10 min of exposure,

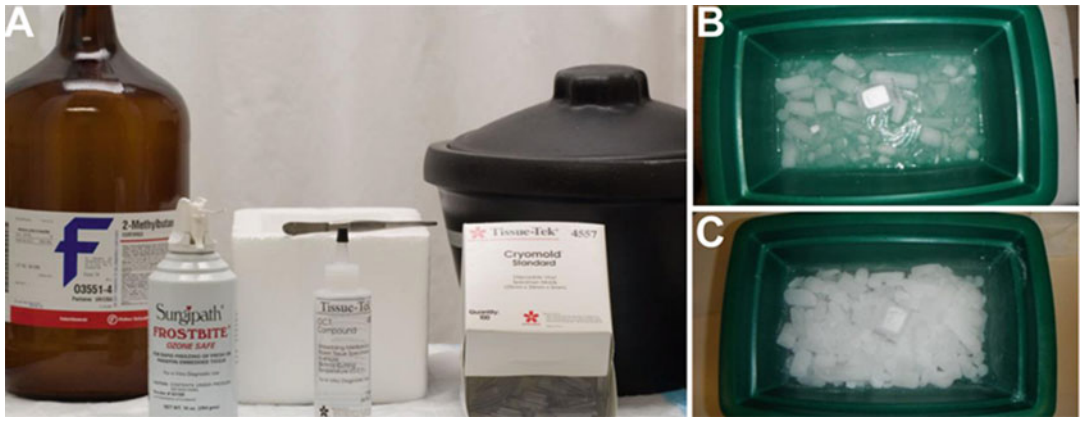

Fig. 1 Tissue embedding in Tissue-Tek® OCT Compound. (**a**) Materials and equipment for embedding. (**b**) Incubation of cryomold with tissue embedded in OCT on the slurry of dry ice and 2—methylbutane. (**c**) Solidified block is transferred to dry ice for complete evaporation of 2-methylbutane prior to storage

observe the animal for signs of unconsciousness (lack of respiration and pedal reflex, faded eye color).

5. Subject animal to bilateral thoracotomy, secure the animal with the pins to the dissecting board, and blot any blood with kimwipes (*see* **Note 7**).
6. Wet the mouse with 70% ethanol, cut the abdominal skin open, and pin it to the board along the body to avoid contamination of target organs with hair (*see* **Note 8**).
7. Use separate RNase-free instruments for dissection of each organ type (*see* **Note 9**).
8. Embed each organ in OCT media on slurry of dry ice and 2-methylbutane (Fig. 1), positioning the organ in the middle of the cryomold (*see* **Note 10**).
9. Make sure to embed and freeze the target organs within 6 min after euthanasia [11].
10. Keeping bags on dry ice, seal prepared OCT blocks in a plastic bag with a sealer, or place them in a double Ziploc bag and transfer to the −80 °C storage prior to cryosectioning (*see* **Note 11**).

3.2 Cryosectioning of OCT Embedded Samples for LCM Slide Preparation and RNA Quality Control in OCT Blocks

During cryosectioning, RNase-free conditions were maintained throughout the procedure, as described previously [11]. Cryosectioning was performed in two steps. *In the first step*, sections were cut from each block for RNA quality control in the prepared tissue block (three replicates per block) (*see* **Note 12**).The last section from each block was mounted on glass slide and stained with hematoxylin and eosin (H&E) for initial pathology evaluation. In addition, for the pilot study, we cut ten serial sections from one random block of normal tissues (mammary gland and lung), primary tumor and lung metastasis. Section number six was mounted on glass slide and stained with H&E for LCM target reference, and the remaining sections were mounted on frame membrane slides for LCM target dissection. *In the second step*, we cut an estimated number of sections per tissue block and mounted them on frame membrane slides for LCM target dissection.

1. Label frame membrane slides with solvent resistant adhesive labels on the "window" side, and incubate in a UV chamber at 352 nm for 30 min for RNase-free conditions and better adherence of frozen sections to the membrane.
2. Prepare lab marker pen, RNase-free SupportSlide, and a box with dry ice lined up with kimwipes. Place labeled Five-Slide Mailers on dry ice inside the box.
3. Set the cryostat at −16 °C for the object and −17 °C for the chamber.

4. Wipe all the surfaces inside and outside the cryostat, brushes, forceps and pencils with 100% ethanol, place three labeled 1.5 ml nuclease-free micro-centrifuge tubes in a small container with dry ice inside the cryostat.
5. Use a new blade for each tissue block.
6. Move OCT blocks, one at a time, from dry ice into the cryostat chamber, and wait for 30 min before mounting the block on the chuck (*see* **Note 13**).
7. Place a small amount of OCT on the chuck, and immediately mount the tissue block. Wait for 10 min prior to sectioning.
8. Face the block, cut three 10 μm sections and put them in a tube kept on dry ice inside the cryostat chamber. Move the tube into the box with dry ice (*see* **Note 14**).
9. Cut and mount the next section on a glass slide to be used as initial H&E. Cut the required number of serial sections for LCM, mounting them on sequentially labeled frame membrane slides, and mounting each 6th section in the sequence on a glass slide. Prepare two extra frame membrane slides to be used as test slides for the setup of laser and camera parameters during laser dissection (*see* **Note 15**).
10. Prechill frame membrane slide in the cryostat chamber for 2 min, cut the section, insert SupportSlide into the "window" of frame membrane slide (Fig. 2), and mount the section onto the

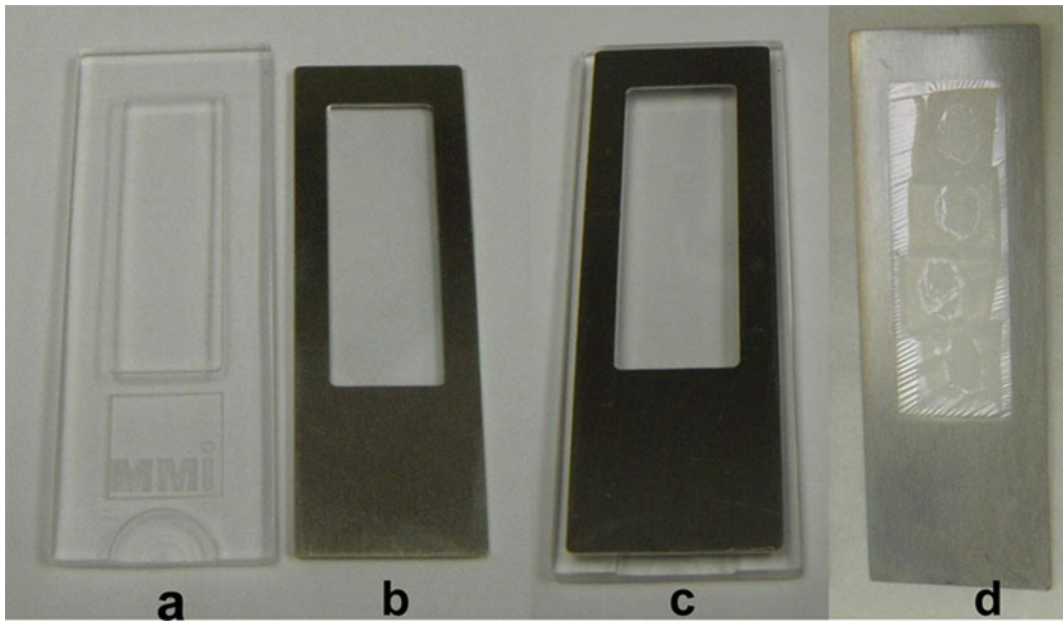

Fig. 2 Sectioning of OCT tissue blocks for LCM. (**a**) MMI SupportSlide, (**b**) Frame membrane slide, (**c**) SupportSlide and frame membrane slide assembly, (**d**) Multiple serial sections mounted on frame membrane slide after trimming of OCT block

membrane. Remove SupportSlide and put frame membrane slide on dry ice always keeping the lid on the box. Keep SupportSlide at room temperature (RT) for mounting the next section.

11. After the acquisition of a required number of slides for the sample, detach the block from the chuck, wrap in aluminum foil and return to the Ziploc bag on dry ice. Transfer the slides into the appropriately labeled Five-Slide Mailers (*see* **Note 16**) and place them in a Ziploc bag.
12. Transfer the slides and blocks to −80 °C storage out of the box with dry ice.

3.3 Determination of RNA Quality in Tissue Samples Collected for LCM

1. Prepare all the required components of AllPrep® DNA/RNA Micro Kit for RNA extraction.
2. Transfer the tubes with frozen sections from −80 °C storage into the box with dry ice.
3. Working with each tube one at a time, warm up the tube lid with your fingers keeping the tube in dry ice. Open the lid, add 350 μl of buffer RLT to the frozen sections, vortex the tube for 2 min at maximum setting, then place in wet ice. Process the rest of tubes for the entire extraction batch in the same manner.
4. Transfer the tubes from wet ice to the tube rack and incubate for 5 min at RT.
5. Vortex the tubes for 30 s and proceed with total RNA extraction according to the manufacturer's instructions.
6. Determine RNA concentration in the samples using NanoDrop.
7. Dilute the samples with RNase-free water to the concentration below 5000 pg/μl and load them on Agilent PicoChip.
8. Determine RNA Integrity Number (RIN) in RNA samples based on Agilent Bioanalyzer run [16].

3.4 Digital Images of H&E Slides for LCM Pathology Annotation Reference

1. Stain sections on glass slides with H&E stain (*see* **Note 17**).
2. Scan the slides on Aperio Scan Scope®XT scanner and transfer images into the study folder for annotation of LCM targets by a study pathologist.
3. Record image IDs of the sections containing the following targets—EMT, MET, carcinoma, lung metastases, normal mammary gland and normal lung parenchyma—for quick reference during LCM.

3.5 Staining of Frame Membrane Slides for Laser Dissection

Staining of LCM slides was conducted in the fume hood under RNase-free conditions in 50 ml conical polypropylene tubes, containing 45 ml of solution, one slide at a time, as previously described [11].

1. Change the solution after each batch of six slides.
2. Prepare the following items: (a) solution of 3% glacial acetic acid in 100% ethanol—place in Styrofoam box with dry ice for 1 h, (b) MG with ProtectRNA —add 4 μl of ProtectRNA to 1 ml of MG, mix by vortexing, (c) Cresyl violet acetate/eosine Y mix (CVAE) —combine 75 μl of 1% alcoholic cresyl violet acetate solution, 25 μl of eosin Y, 250 μl of RNase-free water and 250 μl of 100% ethanol, mix by vortexing, (d) 100% ethanol, (e) xylene, (f) desiccator with desiccant.
3. When fixative is chilled, transfer the LCM slide from dry ice into the tube with fixative on dry ice and incubate for 30 s.
4. Apply 1 ml of MG/ProtecRNA mix to the slide using barrier pipette tip, and incubate for 20 s. Drain the slide and repeat the procedure.
5. Rinse the slide in 100% ethanol for 10 s with up and down movement.
6. Apply 300 μl of CVAE to the slide using barrier pipette tip and incubate for 30 s.
7. Dehydrate the slide in two changes of 100% ethanol for 30 s each.
8. Incubate the slide in two changes of xylene for 2 min each.
9. Dry xylene off the slide in a fume hood and place it in a desiccator at least for 15 min prior to laser dissection (*see* **Note 18**).

3.6 Laser Microdissection and Preparation of LCM Lysates for NanoString Analysis

Dissections were performed on MMI CellCut Plus instrument at 10× magnification with UV laser, within 30-min dissection time per slide, following RNase-free conditions.

1. Power the MMI Cell Cut Plus instrument and start the software.
2. Power the on-network computer, start Image Scope™ software, open the folder with annotated images, and upload the selected image on the screen.
3. Wipe the base of the regular dissecting microscope with RNase AWAY.
4. Prepare the following items for the LCM session: (a) forceps, (b) Styrofoam box with wet ice, (c) Styrofoam box with dry ice, (d) RNase-free glass slides, (e) RLT buffer, (f) PCR tubes filled with mixture of 5 μl of buffer RLT, prepared according to the manufacturer's instructions, and 0.3 μl of RNaseOut, (g) IsolationCaps, (h) RNase-free barrier tips for PT-20 pipette with the tip cutoff to a 3 mm stump with the barrier, (i) stained test frame membrane slides, (j) 100% ethanol.
5. Place the tubes with lysis buffer in wet ice.

6. Load the glass support slide on the dissecting stage and position test slide on the top of the glass slide, with the slide label facing up. Make sure that the sandwiched slides leveled inside the holder.
7. Select 4× objective and set up slide limits in the software tool panel.
8. Change objective to 10×, bring tissue in focus, adjust illumination with the camera controls, and adjust laser focus and line width with power and speed sliding controls (*see* **Note 19**).
9. Disable "AutoShape" and "Collect with Cap UP" function, and select "Auto New Cap."
10. Change to 4× objective, remove the test slide together with the glass slide, and insert the new glass slide and the first serial frame membrane slide stored in a desiccator. Start the timer set for 30 min.
11. Create an overview of a tissue section and change to 10× objective.
12. Load IsolationCap in the cap lift, select "Cap Up position."
13. Using Image Scope™ image registration software match the position and magnification of H&E reference slide on the overhead reference monitor to the image of the tissue on LCM dissecting screen.
14. Using slide overview, move the area of the section with the first target in the middle of the dissecting screen, and reflect the annotations from Aperio image to the dissecting screen with the drawing touch pen.
15. Check the laser position, press the "Cut" button and collect the target cutout with the IsolationCap.
16. Collect the rest of the targets, overlapping cutouts on the IsolationCap (Fig. 3) and stop collection when the timer goes off (*see* **Note 20**).

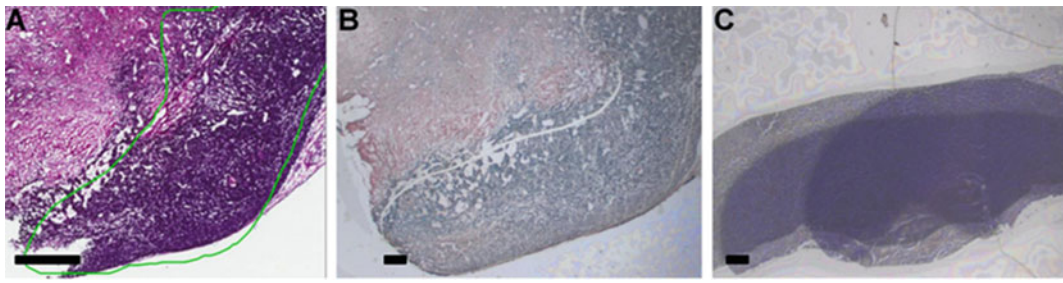

Fig. 3 LCM target collection for NanoString analysis with "Auto New Cap" setup. (**a**) Target area annotated by study pathologist on a digital image of reference H&E section of mammary gland. (**b**) Dissecting screen view of the target area with the laser cut path. (**c**) View of LCM cap with three overlapped collected targets. (**a**) Scale bar corresponds to 650 μm; (**b**, **c**) Scale bars correspond to 300 μm

17. Remove the cap from the cap lift and transfer it under the conventional dissecting microscope with 1.5× magnification.
18. Detach the overlapped tissue in bulk from the IsolationCap with fine tip forceps and transfer it to the PCR tube with lysis buffer (*see* **Note 21**).
19. Vortex the tube for 15 s, spin down, check that the cutouts are submerged in lysis buffer, and return the tube on ice.
20. Repeat dissections for the next serial slide adding the cutouts to the same tube with lysis buffer until the collection of required amount of cutouts is completed. Then incubate the tube at RT for 20 min, vortex for 30 s, and spin down (*see* **Note 22**).
21. Insert the stump of the barrier pipette tip into the new PCR tube, and transfer the lysate onto the filter barrier. Spin down the tube with the remaining lysate, gather cutouts in a lump with the pipette tip and transfer them to the bottom of the filter barrier not touching the sides (*see* **Note 23**).
22. Centrifuge the filter-tube assembly at 6000 × *g* for 30 s, and repeat as needed until all the lysate passed through the filter barrier. Discard the stump with cutouts.
23. Measure the lysate volume and adjust it with original lysis buffer to 4.5 μl.
24. Place the tube on dry ice and transfer to −80 °C storage up to 4 weeks prior to NanoString analysis (*see* **Note 24**).

3.7 Pilot Study for the Determination of RNA Quality and Content in LCM Targets

We found out that a pilot study is an important part of any LCM project that needs to be tailored to the tissue type. We selected a block with the largest target areas from the set of samples dedicated to the LCM study and cut 10–20 extra sections prior to final LCM of the whole set. These sections were used for: (a) evaluation of LCM workflow design for preservation of RNA integrity in LCM targets, (b) evaluation of approaches to sample collection and preparation, (c) estimation of RNA content per area of LCM target, (d) estimation of target area/number of sections/slides needed to satisfy the requirements of the downstream input.

1. Based on annotation data in Aperio Image Scope software, calculate the combined areas of several annotated targets (in mm^2) for each of three replicates for RNA isolation, and make sure that each set of selected targets can be dissected and lysed within 30 min.
2. Perform LCM and prepare lysates as describes in Subheading 3.6.
3. Add 350 μl of buffer RLT to the Nanostring lysate, vortex and put the tube on dry ice.

4. Proceed with RNA extraction, quantification and RNA quality assessment as described in Subheading 3.3 (*see* **Note 25**).
5. Calculate RNA yield in ng/mm^2 of the target tissue as an average of three replicates. Adjust the number upward by 30% for the typical loss of RNA in column based extraction methods. Use this value for the estimate of target area (in mm^2) which is required for the acquisition of 100 ng of total RNA (*see* **Note 26**).
6. Conservatively estimate the number of slides needed for LCM of each target, based on the estimate of target area size and the annotation data of reference H&Es (*see* **Note 27**).

3.8 NanoString Analysis of LCM Samples

1. Select genes of interest together with housekeeping genes for data normalization to create the Nanostring Probe Set for each gene of interest (Reporter CodeSet and Capture ProbeSet) for the nCounter® Custom Gene Expression Assay, or acquire the commercially available pathway panel of interest (NanoStrings Technologies, Seattle, WA) (*see* **Note 28**).
2. Create a master mix containing 130 μl of the Report CodeSet and 130 μl of hybridization buffer, and add 20 μl of each solution into the prepared screw cap centrifuge tubes.
3. Add 5 μl of LCM RNA lysate to each tube, and immediately before placing the reaction at 65 °C, add 5 μl of Capture ProbeSet to each tube following the nCounter Gene Expression Assay Manual instructions.
4. Incubate hybridization assays for at least 12 h, and, following the manufacturer's instructions, immediately proceed to post-hybridization processing with the nCounter Prep Station which detects the targets with molecular barcodes.
5. Collect and tabulate the data by the Digital Analyzer.
6. Using nSolver Analysis Software, import the RCC file with the nCounter data.
7. Make sure that the completed run satisfies the quality control requirements of NanoString platform: (a) Imaging performance—600 FOV (fields of view per sample), (b) sample saturation-binding density between 0.05 and 2.25 across the samples, (c) positive controls—linearity with corresponding dilutions, (d) negative controls—values in a range from 0 to 10 (*see* **Note 29**).
8. Proceed to normalization of the data: (a) correct the raw counts by subtracting the average value of negative controls, (b) normalize the data by the average values of the set of housekeeping genes using nSolver Analysis Software.

9. Perform ANOVA test between normalized data attributed to different LCM cell populations with chosen p-value and fold change value (*see* **Note 30**).
10. Use unsupervised hierarchical clustering method for grouping and segregation of samples (*see* **Note 31**).

4 Notes

1. Necropsy protocol should be designed in a way that the target tissue would be stabilized by freezing in 6 min after animal death [17, 18].
2. Materials from the unopened packaging are RNase-free; with consequent use, avoid materials exposed to the environment. Tools can be cleaned for RNase-free conditions with RNase AWAY and rinse in nuclease-free water.
3. It is important to have clear identification for collected samples. Hand writing on the plastic molds with a laboratory marker is not recommended due to instability of the marker ink during handling of the sample. It is advisable to affix the adhesive cryo labels to the molds before transferring them to −80 °C.
4. All the samples collected with our approach contained high-quality RNA: RINs 9.6–9.8 from mammary primary tumor and lung metastasis blocks, and 9.1–9.7 from blocks of normal tissues (Fig. 4). Since RNA is subjected to some degradation during staining steps of LCM, it is crucial to have sample of high molecular integrity for LCM experiment for unbiased gene profiling results.
5. Consult with statistician about the sample set size for the study. Considering the cost and length of animal experiments, statistically sound planning is crucial to address sample variation and obtain reliable results.
6. We conducted all animal procedures under conditions approved by the Frederick National Laboratory for Cancer Research, an AAALAC accredited institution that follows the Public Health Service Policy for the Care and Use of Laboratory Animals outlined in the "Guide for Care and Use of Laboratory Animals" [19]; Frederick National Laboratory for Cancer Research ACUC 11-067 approval on 03/16/2012. All the animals were sacrificed with tumor sizes below two centimeters in diameter, and maximum weight loss below 20% of initial total body weight.
7. Blood is enriched with RNases and triggers RNA degradation in contaminated samples. In case of contamination, blood should be blotted off the target organ before embedding it in OCT media.

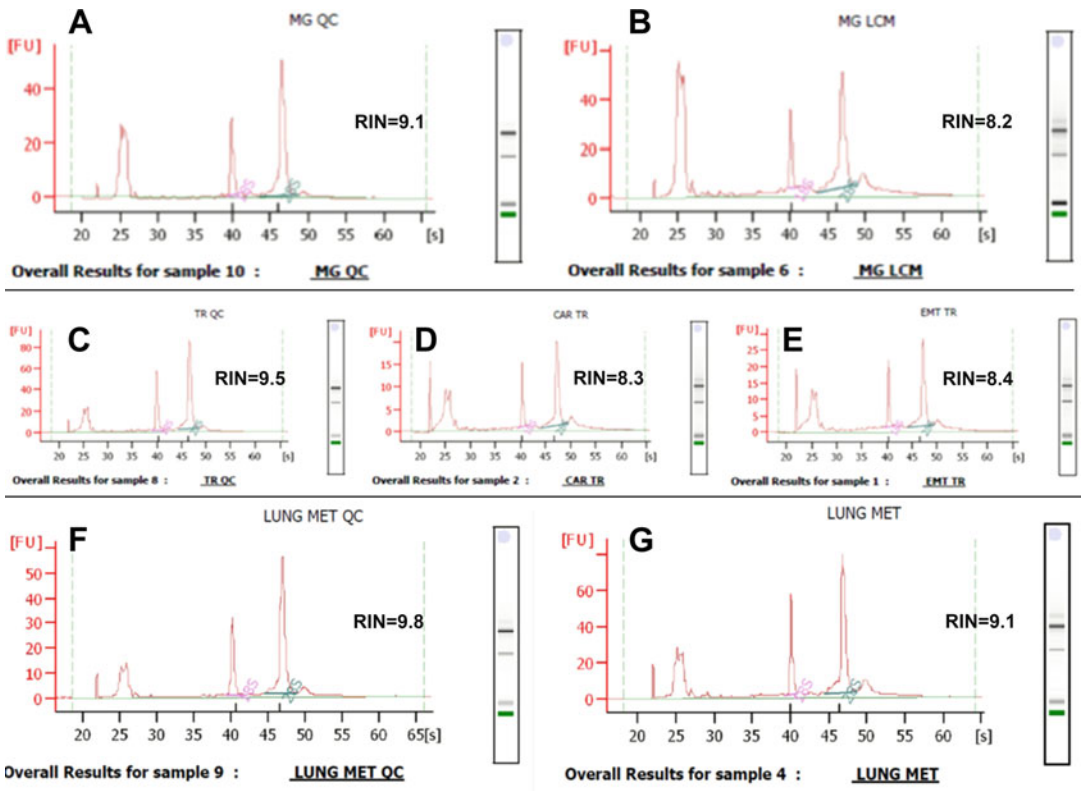

Fig. 4 Representative Agilent electropherograms of high quality RNA retrieved from the control sections and corresponding LCM targets. (**a**, **c**, **f**) Frozen section of normal mammary gland (**a**), primary mammary tumor (**c**) and lung metastasis (**f**) placed directly in lysis buffer for RNA extraction. (**b**) LCM sample of normal mammary tissue. (**d**, **e**) LCM cell populations of primary mammary tumor: carcinoma (**d**) and EMT (**e**). (**g**) LCM sample of lung metastasis (reproduced from Open Access ref. 15)

8. Samples with hair are often unsuitable for LCM because of compromised cryosectioning and laser dissection.
9. Cross contamination of samples compromises molecular profiling especially when the traces of RNA-rich organ are accidentally introduced in the sample with low RNA content.
10. To facilitate mounting of OCT embedded tissue on a slide and provide flexibility for block trimming and facing, the tissue should be positioned in the middle of the cryomold with the thin layer of OCT on the bottom of the mold. Put the mold on slurry and immediately fill it with OCT, avoiding bubbles.
11. We routinely start sectioning for LCM experiments within 2 weeks of sample collection. However, in our experience RNA quality in properly collected, embedded and stored OCT blocks of mouse tissue remains the same up to 4 years from the time of necropsy.

12. Knowledge of initial RNA quality in the tissue is necessary for the evaluation and troubleshooting of sample collection and LCM workflow. Samples of low RNA quality should be excluded from the sample set, or grouped with the other samples of similar quality to avoid biased results in downstream analysis.
13. Thirty minutes of OCT block equilibration to cryostat temperature is crucial for the morphological quality of the section.
14. When cutting extra sections from the block *in the second step*, discard the first 20 μm from the face of the block because the surface layer of tissue exposed to the atmosphere usually has degraded RNA and sub-optimal morphology.
15. Since sectioning *in the second step* is based on the H&E reference slide, the block can be trimmed in a way that facilitates acquisition of multiple sections on the slide (Fig. 2). This approach allows timely collection of a sufficient number of LCM targets on IsolationCap without refocusing the laser.
16. Storing slides for one sample in several Five-Slide Mailers, versus the 25 or 50 slot slide box, allows handling five slides at a time during LCM session and keeping the rest of the slides at constant −80 °C temperature for preservation of RNA integrity. The prepared LCM slides were stored for 2 weeks prior to LCM.
17. We use a modified manual staining protocol for LCM reference sections: air drying -10 min, 10% neutral buffered formalin-10 min, tap water-5 min, dH_2O-1 min, Hematoxylin-2-2 min (filtered), tap water-10 min,70% ethanol-1 min, 95% ethanol-1 min, eosin Y-10 s, 100% ethanol-1 min (four changes), xylene-3 min (three changes). If an autostainer is used for H&E stain, after fixation load the slides on the stainer in distilled water and start from hematoxylin step.
18. Drying in a desiccator improves laser focusing. Slides with sections of primary tumors, lung metastasis and normal mammary gland and lungs did not show any sign of RNA degradation during the 5-h storage in a desiccator prior to dissection [15].
19. We carried all the dissections with laser speed at 37%, laser focus at 78% and laser power at 41%.
20. We prefer to load three serial slides simultaneously, and dissect the targets sequentially from all slides with the same IsolationCap in a 30-min time period. The speed of dissection can be estimated in a pilot study using a test slide.
21. Forceps method works well for the cutouts of 200 μm or larger. Smaller targets should be collected with 0.2 ml IsolationCap. Dislodge smaller cutouts from the cap by the following

procedure: (a) dispense 2 μl of 100% ethanol in the lid of the PCR tube, (b) bring the cutouts on the IsolationCap in contact with the drop of ethanol, (c) observe the IsolationCap under a dissection microscope to make sure that all cutouts were dislodged, (d) close the lid of the PCR tube, centrifuge at 16,000 × *g* for 15 s, then keep the tube on ice, (e) add more dissected targets to the tube in the same manner, (f) at the completion of dissection, evaporate ethanol, add 5 μl of lysis buffer into the tube, and continue with the lysis procedure.

22. Accumulation of dissected targets for 2.5 h in lysis buffer on wet ice did not cause degradation of RNA in any of our samples (Fig. 4). Longer times have not been tested.
23. Small cutouts often obstruct the pipette tip during pipetting, causing variability among input volumes in the NanoString hybridization reaction. Our filtering technique eliminates this problem. Barrier tips for PT-200 pipettes can be used in the same way with 0.5 ml centrifuge tubes.
24. Four-week storage of RNA lysates at −80 °C prior to NanoString analysis and 1 week storage of RNA purified from the whole tissue samples for microarray analysis is acceptable; we observed high concordance of genes selected for the study of spontaneous carcinoma model between the platforms [15].
25. LCM material should be collected and extracted with the minimal elution volume recommended by the manufacturer to measure RNA concentration by NanoDrop. Measurements starting from 20 ng/μl can be reliably used for the estimation of RNA content in LCM targets of the same sample set.
26. It is impossible to obtain uniform (by RNA concentration) LCM samples due to variability during RNA extraction; the average of three technical replicates of RNA content per mm^2 can differ by 30–40%. However, such a range among the samples is accounted for by the normalization algorithm of the NanoString platform [13].
27. On some occasions, the dissected annotated target area may be larger than required to obtain 100 ng of RNA. In these cases, increase the default volume of lysis buffer (5 μl) proportionally to the size of the dissected area calculated by MMI Cell Cut Plus Software prior to sample incubation and cutouts removal. Monitor the sum of dissected areas in MMI software during LCM of serial sections. If possible, collect more material then needed because it is easier to work with volumes larger than 5 μl during lysis and following the removal of cutout membranes from the tubes. We prefer adding on average, two to four extra sections to the estimated number. The excess of RNA lysate can be used for other types of analysis.

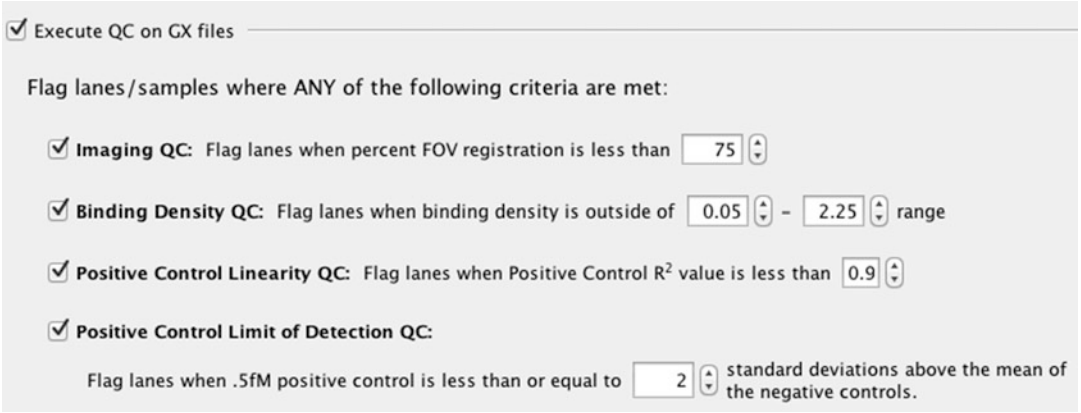

Fig. 5 nSolver QC parameters. The table represents numerical parameters used in this study to access LCM samples quality control for NanoString gene profiling (reproduced from Open Access ref. 15)

28. We selected 103 mRNA genes and controls classified as embryonic stem cell (ESC), epithelial–mesenchymal transition (EMT), and mesenchymal—epithelial transition (MET) markers based on published data [15].
29. All the LCM samples in our study satisfied imaging quality control metrics for nCounter data analysis (Fig. 5).
30. An ANOVA test conducted between LCM samples of epithelial-like cells (6 carcinoma samples) and spindle-like (8 EMT samples) cells identified 17 differentially expressed genes (fold change 1.5 and p-value <0.05) [15].
31. The unsupervised hierarchical clustering was based on the expression pattern of epithelial-like cell gene sets and spindle-like gene sets. The clustering reveled two main branches, segregating the samples into distinct groups (Fig. 6); overexpression of *Klf4*, *Wt1* and *Msi1* was observed in the EMT (spindle-like areas), whereas overexpression of the *Epcam*, *T*, *Foxc2*, and *CD24a* was observed in carcinoma areas [15].

Acknowledgments

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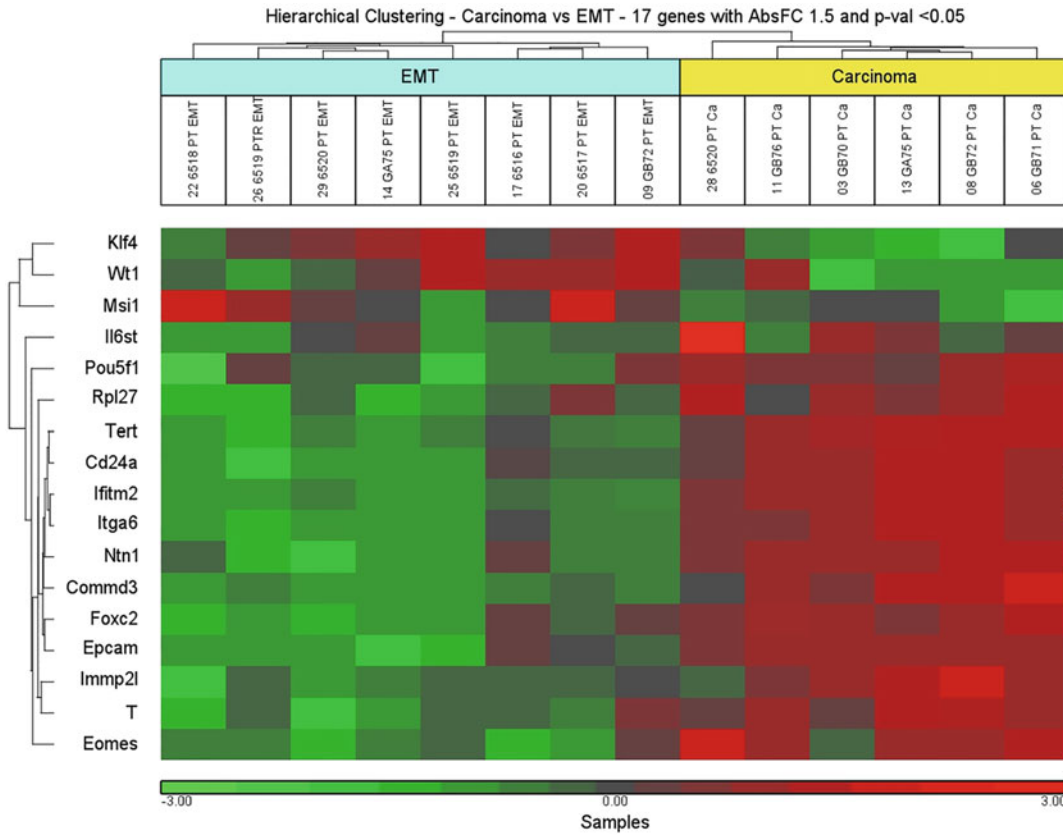

Fig. 6 Unsupervised hierarchical clustering of carcinoma versus EMT. Scaled down representation of the entire cluster is based on 17 genes differentially expressed between carcinoma (epithelial-like areas) and EMT (spindle-like areas). Each row represents a single gene and each column represents a sample. Red color indicates upregulation, green color—downregulation, and black color—no change in expression level, compared with the reference sample. EMT: Epithelial–Mesenchymal transition (reproduced from Open Access ref. 15)

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Chapter 7

Laser Capture Microdissection as a Tool to Study the Mucosal Immune Response in Celiac Disease

Giuseppe Iacomino, Vera Rotondi Aufiero, Pasquale Marena, Antonella Venezia, Riccardo Troncone, Salvatore Auricchio, and Giuseppe Mazzarella

Abstract

Laser capture microdissection (LCM) is a powerful tool for selection and isolation of single cells or compartments from complex primary tissues to perform molecular analyses. Celiac disease is a genetic autoimmune disorder where the ingestion of gluten leads to damage in the small intestine. Increased intraepithelial lymphocytes and the presence of the lamina propria inflammatory infiltrate of the duodenal mucosa is a common part of the disease. These cells promote inflammatory processes through the release of cytokines. Here, we describe the use of LCM and real-time quantitative PCR (RT-qPCR) to analyze cytokine profile information in distinct duodenal mucosa tissue compartments of celiac patients.

Key words Laser capture microdissection, RT-qPCR, Intestinal compartments, Cytokines, Celiac

1 Introduction

Over the past decades, there has been a revolution in understanding the molecular pathogenesis of human diseases. RNA research has made a significant impact on all aspects of biomedical research, from clarifying basic cell functions, mapping involved pathways to understanding gene expression changes, producing new promises for molecular diagnostics and basic science research. A development has been the implementation of relatively new technologies that permit analysis of gene expression in a limited number of cells, virtually in a single-cell type. Laser capture microdissection (LCM) is a contact- and contamination-free method for isolating specific single cells or entire areas of tissue from a wide variety of tissue samples. The dissectate is then available for further molecular biological methods such as PCR, real-time PCR, proteomics, and

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other analytical techniques [1]. LCM has had a major impact in a large number of research fields, e.g. cancer, neurological diseases, infectious diseases, and immunological disorders [2], providing new insights into both normal cell biology and pathogenic mechanisms.

Celiac disease (CD) is a T-cell-mediated immune disease in which gliadin-derived peptides activate lamina propria effector CD4+ T cells. This activation leads to the release of cytokines, which play a crucial role in the pathogenesis of CD, controlling many aspects of the inflammatory immune response [3]. Different approaches have been used to study the pattern of cytokines in CD, which have led sometimes to contradictory results. Most data has been found by mRNA expression in biopsy homogenates [4–6] from patients with active CD. However, the results of analysis of whole tissue samples are usually determined by the major or predominant cell type and may mask biologically relevant and important changes present either in the different tissue compartments or in a particular type of cells. Other studies analyzed cytokine mRNA expression in enterocytes, intraepithelial lymphocytes (IELs), and lamina propria mononuclear cells (LPMC), obtained from intestinal biopsy specimens of CD patients, by using techniques that fail to provide an isolation of highly pure cell populations [7–9]. Therefore, it is essential to analyze specific tissue compartments or cell types to identify and define biologically important processes. The development in the last decade of LCM has allowed this goal to be achieved by combining microscope-based morphological methods of analysis with a diverse range of very powerful molecular technologies [10–12].

The recovery of high-quality RNA or other macromolecules from microdissected tissue depends on several parameters, such as quality, fixation and storage of tissue biospecimens, as well as processing time for staining and microdissection [13–15].

Therefore, the purpose of this chapter is to provide tips and tricks to perform LCM for the isolation of tissue compartments from frozen celiac duodenal mucosa and obtain sufficient amounts of high-quality RNA for cytokine gene expression profiles by RT-qPCR [16].

2 Materials

2.1 Tissue Sectioning, Staining, and Laser Microdissection

1. Arcturus PEN membrane Glass Slides (Applied Biosystems).
2. Cryostat (Leica CM1850; Leica Microsystems, Wetzlar, Germany).
3. Cryostat blades, carbon steel C35 (Feather safety razor, LTD. medical division).
4. Paintbrushes with 0.5–1 cm stiff bristles.

5. RNase-free 0.5 mL PCR tubes (Eppendorf, Hamburg, Germany).
6. RNase*Zap* (Applied Biosystems, Carlsbad, CA, USA).
7. Nuclease-free water (Applied Biosystems).
8. 70%, 95%, and 100% ethanol (EtOH).
9. Xylene.
10. Diethyl pyrocarbonate (DEPC, Sigma).
11. Tissue-Tek Cryomold Standard, 10 × 10 × 5 mm (Electron Microscopy Sciences, Hatfield, PA).
12. Cryomatrix optimal cutting temperature (OCT) compound (Thermo Fisher Scientific).
13. RNase-free Conical Tubes 50 mL (Thermo Fisher Scientific).
14. Mayer's haematoxylin.
15. 4 U RNasin RNase inhibitor (Promega, Italy).
16. Microscope plastic Slides Mailers Box for holding up top to two slides (Leica Biosystems).
17. Leica LMD 6000 microdissector.

2.2 RNA Preparation from LCM Samples, Quality, and Quality Assessment

1. PicoPure RNA Isolation Kit (Arcturus®, Thermo Fisher Scientific).
2. RNaseZap Solution (Ambion, Thermo Fisher Scientific).
3. Nuclease-free water (Applied Biosystems, Thermo Fisher Scientific).
4. RNasin RNase inhibitor (Promega).
5. 1.5 mL PCR tubes (Eppendorf).
6. 100% EtOH.
7. 100% Isopropanol.
8. 2100 Bioanalyzer (Agilent Technologies) or Experion (Bio-Rad Laboratories) with chip priming station.
9. Agilent RNA 6000 Pico Assay Kit or Biorad RNA HighSens Analysis Kit.
10. RNase-free 1.5 mL snap cap microcentrifuge tubes.
11. Pipette accurate for measuring 1–10 μL volume.
12. Qubit RNA HS Assay Kit (Thermo Fisher Scientific).
13. Qubit Fluorometer (Thermo Fisher Scientific).
14. Thermo Block.
15. Refrigerated centrifuge.
16. RNase-Free DNase Set (Qiagen).

2.3 cDNA Synthesis and Real-Time Polymerase Chain Reaction

1. SuperScript VILO cDNA Synthesis Kit (Invitrogen, Thermo Fisher Scientific).
2. Power SYBR Green PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific).
3. Custom primers (Thermo Fisher Scientific).
4. Nuclease-free water.
5. Thin-walled, RNase-free PCR optical tubes or plates (Applied Biosystems, Thermo Fisher Scientific).
6. Thermocycler: ABI PRISM 7000 Sequence Detection System, 7900HT Fast Real-Time PCR detection System, or QuantStudio 7 Real-Time PCR (Applied Biosystems, Thermo Fisher Scientific).

3 Methods

3.1 General Precautions

1. Generally, frozen tissues are better than paraffin embedded tissue to preserve RNA. Importantly, high-quality RNA can be achieved from snap-frozen tissue in liquid nitrogen immediately after the surgical excision (*see* **Note 1**).
2. All equipment and laboratory benches should be thoroughly cleaned with RNase*Zap* and then rinsed with nuclease-free water or deionized water.
3. All pipette tips, tubes, reagents, and other consumables must be RNase-free.
4. Pipette tips should contain barriers and should be changed each time you pipette, even if you are pipetting the same reagent, to avoid potential cross-contamination between samples and to prevent RNase contamination.
5. For most procedures, it is advisable to use nuclease-free, hydrophobic, nonstick tubes to minimize loss of sample that may otherwise adhere to the tube walls.
6. Gloves should be worn at all times and changed frequently, especially after coming into contact with liquids or surfaces that may be contaminated with RNases.
7. To avoid contaminations that could degrade RNA, all solutions (water and other solutions) should be treated with 0,1% DEPC-treated-autoclaved-water (*see* **Note 2**).

3.2 Sectioning and Staining

To prevent RNA degradation, tissue sectioning and staining must be performed as quickly as possible (within 15 min) processing one slide at a time.

1. Spray PEN membrane glass slides with RNase*Zap*, wash with Nuclease-free water, and then incubate the slides in a UV chamber for 30 min.

2. Sterilize cryostat Blade with UV radiation for 20 min or by autoclaving for 20 min.
3. Under a hood clean the cryostat blade holder with 100% EtOH and treat the brushes that will be used to manipulate the tissue sections with RNase*Zap*.
4. Set the cryostat temperature at −26 °C.
5. Cool the specimen brushes and the blade in the cryostat.
6. Spray microscope plastic slides mailers box with RNase*Zap* and allow them to sit for 10 min. Rinse twice with distilled water and then perform a final rinse with nuclease-free water. Allow the containers to dry under a hood.
7. Prepare, in RNase-free conical tubes, 50 mL of 70% EtOH and 50 mL of 95% EtOH solutions, diluting 100% EtOH with 0.1% DEPC treated water.
8. Fill individual microscope slides box with the above EtOH solutions, xylene and DEPC water, and hold them on cold ice.
9. Inside the cryostat, remove the frozen OCT-embedded tissue from its cryomold and mount securely to the metal specimen stage with OCT compound.
10. Using a fresh disposable blade, shave OCT from the block until the tissue becomes visible. Set the cutting thickness to 9 μm (*see* **Note 3**).
11. Section the tissue and use a small brush to straighten out the newly cut sections.
12. Straighten out the newly cut sections by using a small brush and mount onto a PEN membrane glass slides (*see* **Note 4**).
13. Allow the slide to air-dry for 2 min under a hood (*see* **Note 5**).
14. Fix the slide in ice-cold 70% EtOH with shaking for 2 min (*see* **Note 6**).
15. Rinse the slide with ice-cold DEPC water with shaking for 60 s.
16. Add a drop of Mayer's Ematoxilyn on each section and incubate for 15 s (optional, *see* **Note 7**).
17. Rinse the slide with ice-cold DEPC water with shaking for 30 s.
18. Rinse the slide with ice-cold 70% EtOH, with shaking for 30 s.
19. Rinse the slide with ice-cold 95% EtOH, with shaking for 15 s.
20. Rinse the slide twice with ice-cold 100% EtOH, with shaking for 30 s.
21. Rinse the slide twice with ice-cold xylene with shaking for 60 s.
22. Allow the slide to air-dry at least 5 min before LCM.

Change the blade cutting surface and each solution between each subject's sample.

3.3 Laser Capture Microdissection

To avoid RNA degradation, microdissection must be finished within 30 min processing a single compartment at a time (*see* **Note 8**).

1. LCM experiment was performed using an LMD6000 Laser Microdissection system.
2. Add 29 μL of Extraction buffer from Arcturus Pico Pure RNA Isolation kit (*see* **Note 9**) and 1 μL of RNase Inhibitor (40 U/μL) (*see* **Note 10**) into the cap of a clean 0.5 mL Eppendorf tube. Place the cap into the cap holder apparatus of the laser microdissection system (*see* **Note 11**).
3. Load the slide on the slide holder of Leica Microsystems LMD systems support and put it onto the microscope stage.
4. Using the joystick place the sample slide onto the stage.
5. Utilizing a 20× objective, select the cutting area from the compartment of interest, with the help of the mouse by drawing a line around each one of them.
6. Select the cap at the load line position.
7. Set laser parameters (*see* **Note 12**) and then activate laser clicking on "START CUT" button. Specimen microdissected out of tissue slides falls into the cap of the selected microcentrifuge tube via gravity.
8. Move the stage with the joystick and continue firing laser to collect all required material (*see* **Note 13**).
9. To observe captured cells, remove the slide from stage clicking on "Collector" button that gently places the cap in the center of the field of vision. Adjust the fine focus on a microscope and examine the cap to see the collected samples.
10. After microdissection is completed, click on "Unload" button and remove the cap from the cap holder apparatus of the laser microdissection system.
11. Remove and close the tube containing the microdissected area from the holder apparatus, placing it with cap down on ice cold immediately.

3.4 RNA Pre-extraction Step

1. Place the tube containing the microdissected area with cap down in a thermoblock at 42 °C for 30 min.
2. Remove microdissected sample from the incubator. Invert the microcentrifuge tube and spin at 800 × *g* for 2 min to collect cell extracts.
3. Keep the tube on ice if you quickly proceed with the RNA isolation step or alternatively store at −80 °C.

3.5 RNA Isolation

1. To pre-conditioning the RNA Purification Column, dispense 250 μL Conditioning Buffer (CB) onto the assembled purification column and incubate for 5 min at room temperature (RT). Then, spin at 16,000 × *g* for 1 min.

2. Once thawed, mix the microdissected tissue by pipetting twice.
3. Add 30 μL of 70% EtOH into the microdissected sample and accurately mix by pipetting WITHOUT CENTRIFUGING. This results in a 1:1 volume ratio of 70% EtOH: microdissected tissue (the combined volume is approximately 60 μL).
4. Transfer the mixture into the preconditioned column. Gently centrifuge at 100 × *g* for 2 min (RNA binding to the column membrane) and immediately spin at 16,000 × *g* for 30 s to eliminate the flow-through.
5. Add 100 μL Wash Buffer 1 (W1) into the purification column and spin at 8000 × *g* for 1 min (**steps 6–8** are optional, *see* **Note 14**).
6. Pipette 5 μL DNase I Stock Solution to 35 μL of the provided Buffer RDD and gently mix.
7. Pipette the 40 μL DNase mix into the purification column and incubate at 37 °C for 30 min.
8. Pipette 40 μL PicoPure Buffer W1 into the purification column and spin at 8000 × *g* for 15 s.
9. Dispense 100 μL Wash Buffer (W2) into the purification column and centrifuge at 8000 × *g* for 1 min. Make sure that the discarded flow-through does not overtake the purification cartridge.
10. Pipette 100 μL Buffer W2 into the purification column and centrifuge at 16,000 × *g* for 2 min. Additionally, remove any wash buffer residual by re-centrifuging at 16,000 × *g* for 1 min.
11. Remove the working column cartridge and transfer into a new collection tube provided by the kit Supplier.
12. Gently dispense an appropriate volume (*see* **Note 15**) of Elution Buffer (EB) directly onto the surface of the membrane of the purification column to guarantee an optimal buffer distribution, and keep for 1 min at RT.
13. In order to consistently distribute the EB, centrifuge the column at 1000 × *g* for 1 min.
14. Collect total RNA by spinning at 16,000 × *g* for 1 min.
15. The isolated total RNA is now ready and may be immediately used (*see* **Note 16**). Be sure to aliquot samples into volumes appropriate for the quantitative (1 μL) or qualitative (2 μL), analysis and the downstream Reverse Transcription reaction step.

3.6 RNA Quantitation

Following RNA extraction, the measurement of RNA quantity is discretionary but recommended. Keep in mind that, because of the very small RNA amounts obtained from LCM samples, it is often

hard to balance RNA differences across samples prior to RT-qPCR analysis. We found very advantageous to engage the Invitrogen™ Qubit™ 3 Fluorometer due to the small quantity of test sample required using the highly sensitive Qubit® RNA HS Assay Kit (*see* **Note 17**). Moreover, working chemistry employs fluorescent dyes that only produce signal when bound to the target RNA, even at low concentrations, so minimizing the effects of contaminants, including degraded RNA.

A detailed protocol is available online at www.lifetechnologies.com/manuals.

3.7 RNA Integrity Quality Analysis

The integrity of RNA is a very critical aspect regarding downstream RNA-based analysis. RNA quality may be optionally assessed by Lab-on-chip automated electrophoresis stations.

Detailed protocols are available online at:
http://www.genomics.agilent.com/
http://www.bio-rad.com/

1. Preliminarily evaluate concentrations of extracted RNA samples to define the optimal Chip assay to employ (i.e., Agilent RNA 6000 Pico Assay or Biorad RNA HighSens Analysis Kit).
2. Follow the manufacturer's protocol.
3. Evaluate electropherograms analyzing ladder, peaks, and RNA quality indicator value (*see* **Note 18**).

The value of results is directly related to the integrity and purity of RNA template [17]. Use only satisfactory samples.

3.8 Reverse Transcription

In our hands RNAs are reverse transcribed by using the SuperScript® VILO cDNA Synthesis Kit (*see* **Note 19**).

Reactions can be suitably performed in a thermocycler heating block.

1. For a single reaction, mix the following components in a PCR tube on ice. For multiple reactions, it is appropriate preparing an adequate volume of master mix without RNA source to reduce variabilities.

   Components for a single Reverse Transcription reaction (20 μL) are:

(a) 5× VILO™ reaction mix	4 μL
(b) 10× SuperScript™ enzyme mix	2 μL
(c) RNA	up to 11 μL
(d) RNase free water	to 20 μL

2. Gently mix the tube content WITHOUT VORTEXING.
3. Load the reactions into a thermal cycler.

4. Incubate the reaction tube at 25 °C for 10 min.
5. Incubate the reaction tube at 42 °C for 60 min.
6. Terminate the reaction at 85 °C for 5 min.

Generated cDNA may be stored at −20 °C (suitably diluted or undiluted) until use for subsequent processing (*see* **Note 20**).

3.9 Real-Time qPCR Assay

cDNAs are suitably quantified by a SybrGreen-based qPCR approach, with an ABI-PRISM 7000SDS instrument.

All individual cDNA samples and appropriate controls (NTC—No Template Control, no-RT, positive controls) are proficiently amplified using the 2× Power SYBR® Green PCR Master Mix and specific primer/probe sets (*see* **Note 21** and Table 1). All the PCR reaction points are performed at least in triplicate in a final volume of 35 μL (*see* **Note 22**) containing 2 μL of suitable cDNA dilutions (*see* **Note 23**). To assess the specificity of the amplification products analysis of melting curve is required. Serial dilutions of cDNA containing a known quantity of each transcript may be used in each quantitative PCR run to generate a standard curve. Only study samples where both gene and housekeeping gene had a sigmoid-shaped curve between Ct values from 15 to 36 should be usually selected. Samples that do not meet the RNA quality and quantity requirements should be excluded from the study.

3.9.1 Prepare the PCR Reagents Mix

qReal Time-PCR setup should be assembled in a dedicated PCR clean and controlled environment.

1. Allow the 2× Power SYBR Green PCR Master Mix to thaw completely. Mix gently.
2. In a polypropylene tube, prepare the PCR reagents mix by scaling the volumes listed below to the required number of PCR reactions. Include extra volume to account for pipetting losses.

   PCR reagents mix
   Components for a single PCR reaction are:

(a) Power SYBR green PCR master mix (2×)	17.5 μL
(b) Forward primer (20 pmol/μL)*	0.7 μL
(c) Reverse primer (20 pmol/μL)*	0.7 μL
(d) Template	variable
(e) Nuclease-free water	to 35 μL

*Optimal primer concentration must be accurately determined in preliminary assays

3. Mix gently. DO NOT VORTEX. Centrifuge briefly, and then organize the PCR reaction plate.

Table 1
Example of PCR primers used in cytokine profiling of celiac mucosa

Gene	Oligonucleotide sequences (5′ → 3′)		Accession number
	Forward primer	Reverse primer	
IL-10	GCTGGAGGACTTTAAGGGTTACCT	CTTGATGTCTGGGTCTTGGTTCT	*AY029171.1*
IL-15	CCATCCAGTGCTACTTGTGTTTACTT	CCAGTTGGCTTCTGTTTTAGGAA	*U14407*
IL-17a	CAATCCCACGAAATCCAGGATG	GGTGGAGATTCCAAGGTGAGG	*NM_002190*
IL-21	CATGGAGAGGATTGTCATCTGTC	CAGAAATTCAGGGACCAAGTCAT	*NM_021803*
IFN-γ	GTTTTGGGTTCTCTTGGCTGTTA	AAAAGAGTTCCATTATCCGCTACATC	*NM_000619*
TNF-α	CCCCAGGGACCTCTCTCTAATC	GGTTTGCTACAACATGGGCTACA	*NM_000594*
TGF-β1	CAAGGGCTACCATGCCAACT	AGGGCCAGGACCTTGCTG	*NM_000660*
MxA	CAATCAGCCTGCTGACATTG	TGTCTCCTGCCTCTGGATG	*AF135187*
GAPDH	ATGACATCAAGAAGGTGGTG	CATACCAGGAAATGAGCTTG	*NM_002046.2*

Gene expression is normalized to the level of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Study primers are resuspended in nuclease-free water; aliquots are further diluted to 20 pmol/μL working dilutions. Source: adapted from ref. 16

Table 2
Use this 2-step cycling condition for all primers sets with the exception of GAPDH primer pair

Step	AmpliTaq Gold® polymerase activation[a]	PCR (45 cycles)		Melting curve generation
	Hold	Denature	Anneal/Extend	Melt curve
Temp.	95.0 °C	95.0 °C	60.0 °C	
Time	10 min	15 s	1 min	

[a]The 10 min, 95 °C step is required to activate the AmpliTaq Gold® DNA polymerase

Table 3
Use this 3-step cycling condition only for GAPDH primer pair

Step	AmpliTaq Gold® polymerase activation[a]	PCR (45 cycles)			Melting curve generation
	Hold	Denature	Anneal	Extend	Melt curve
Temp.	95.0 °C	95.0 °C	55.0 °C	72.0 °C	
Time	10 min	30 s	30 s	45 s	

[a]The 10 min, 95 °C step is required to activate the AmpliTaq Gold® DNA polymerase

3.9.2 Set Up the Plate Document

See your instrument user's manual for detailed instructions on how to set up the plate document. The thermal-cycling conditions required for determining cytokines reported in Table 1 are described in Tables 2 and 3.

Upon qPCR achievement, data was saved and all acquisitions can be analyzed at a later time. When performing SybrGreen based qPCR assays preliminary:

- explore the absence of nonspecific amplification products by generating a melt curve in your real-time PCR system;
- confirm the expected PCR bands by agarose gel electrophoresis.

3.10 Data Analysis

Methods for PCR data analysis have been described in detailed Reports [18–21] and are beyond the scope of this protocol.

PCR data from this protocol are quantified by a relative quantification. The relative expression of transcripts can be calculated by the ΔΔCT method taking advance from the Data Assist Software v3.0 (Applied Biosystems, Thermo Fisher Scientific) or similar software (GenEx qPCR Data Analysis Software, TATAA Biocenter). A discussion on fold change method can be found in reference [22]. Resulting data can be exported and organized for statistical analysis.

4 Notes

1. In our experience, to recover a high-quality RNA for cytokine gene expression profiling, it is mandatory to snap-frozen tissue in liquid nitrogen immediately after the surgical excision or, at least, within 20 min. Longer times result in increased RNA degradation and low-quality qPCR detection (Fig. 1a).

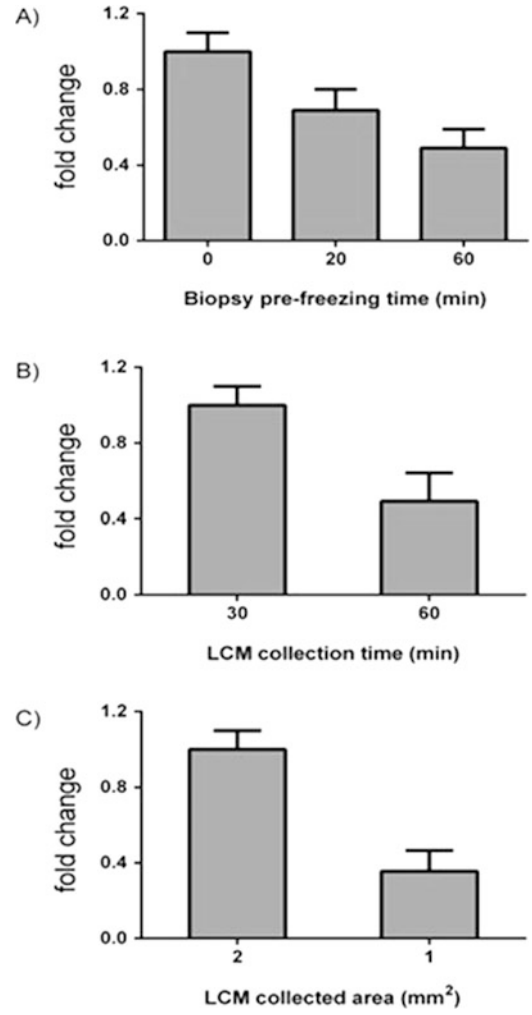

Fig. 1 Test study was undertaken to show the impact of biopsy pre-freezing time (**a**), LCM collection time (**b**), and tissue surface area (**c**) on RT-qPCR assays. LCM samples in panels **a** and **b** were collected from 2×10^6 μm [2] surface area. LCM samples in panel **c** were obtained in 30 min of laser microdissection. Relative expression of transcripts was calculated by the ΔΔCT method on duodenal mucosa. Fold change represents TNF-α messenger RNA normalized to GAPDH. Similar results were obtained with other cytokines. Results are presented as normalized mean expression with SEM

2. Add 1 mL of diethylpyrocarbonate (DEPC) to 1000 mL distilled water. Mix well and let it set at RT for 1 h. Autoclave with high-pressure saturated steam at 121 °C for about 20 min. Let it cool to RT prior to use.
3. In our experience 8–10 μm thick sections are ideal for laser microdissection. Thicker sections are more difficult to cut and fall, while thinner sections may not offer adequate amounts of desired material.
4. Tissues section will not properly adhere to cold slides. Therefore, after sectioning, the cryosections should be picked up on PEN membrane slides kept at RT. Since the first cryosection needs to remain inside the cryostat, place other sections on the same slide within 15–20 s to ensure that they will adhere firmly to the slide. Multiple sections, 6–8, should be picked up on one slide (depending on section size) to obtain an adequate amount of desired material.
5. Perform **steps 13–22** under a hood.
6. OCT can interfere with laser microdissection. We found alcohol 70% more efficient to remove OCT than acetone. If the OCT is not completely dissolved, add fresh 70% EtOH, and wash the slide again with shaking for 30 s.
7. With frozen sections, the different intestinal compartments could be selected without staining of tissue section (*see* Fig. 2), thus shortening the process time.

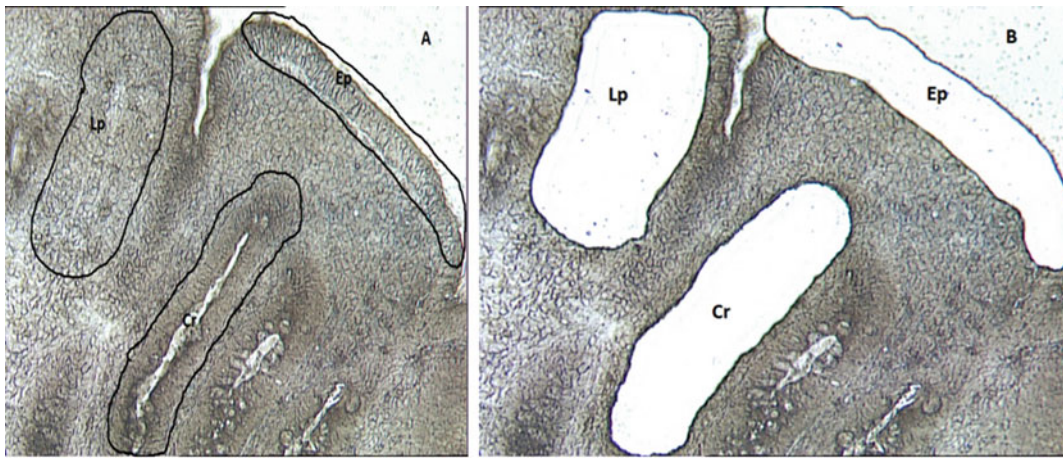

Fig. 2 Selective microdissection of surface epithelium, lamina propria and crypts of Lieberkuhn compartments of frozen section of jejunal biopsy from untreated celiac disease patient. The laser cuts along the line drawn by the operator, isolating the compartments. (**a**) Roadmap before microdissection (Ep indicates the surface epithelium; Lp indicates lamina propria; Cr indicates crypts of Lieberkuhn); (**b**) Postmicrodissection (Ep, Lp anc Cr indicates, respectively, the lifted surface epithelium, lamina propria and crypt of Lieberkuhn compartments). Original magnification ×200

8. In our experience LCM should be performed within 30 min to prevent RNA degradation and improve subsequent detection (Fig. 1b).
9. In our experience the PicoPure RNA extraction kit, a glass filter-based method, provides the largest and most reproducible total RNA yields from microdissected tissue samples.
10. RNAse Inhibitor preserves RNA from degradation and ensures inactivation of endogenous RNases.
11. In our experience, quantities larger than 30 μL may lead to a crystallization of the extraction buffer, thus compromising the yield of RNA extraction.
12. With a 10× eyepiece and a 20× objective, the following parameters are used: aperture, 2; laser power, 40; speed, 8 for clean cuts; offset, 80; specimen balance 6. The preset values are optimized for each objective.
13. We obtained the best results when a total area of 2×10^6 μm [2] was collected (Fig. 1c).
14. DNase optional treatment is highly recommended to reduce the risk of DNA interference in downstream applications potentially sensitive to very small amounts of contaminating DNA. DNase I treatment may be performed directly within the purification column utilizing the RNase-Free DNase Set. Alternatively, move to **step 8**.
15. Recommended elution volume = 11 μL; maximum elution volume = 30 μL. In our hands, RNA is typically eluted in a single 12 μL volume.
16. Alternately, purified RNA may be stored at −80 °C. Under these conditions, no degradation is noticeable up to 6 months.
17. The assay procedure is designed to be accurate for RNA sample concentrations between 250 pg/μL and 100 ng/μL. For downstream application we recommend harvesting a 2×10^6 μm [2] area to obtain approximately 35 ± 5 ng total RNA.
18. Agilent Technologies offers the RIN algorithm (RNA Integrity Number) on the 2100 Bioanalyzer, and Bio-Rad developed an algorithm for calculating the RNA Quality Index (RQI). The RIN and the RQI are based on a numbering system from 1 to 10 (1 being the most degraded RNA profile, 10 being the most intact RNA).
19. Starting material can range up to 2.5 μg total RNA in a 20 μL cDNA synthesis reaction. For probe-based two-step RT-qPCR, linear cDNA yields from 1 pg to 2.5 μg of total RNA in a 20 μL reaction. RT can be used to synthesize cDNA at a temperature range of 42–60 °C. Shorter incubation times

and/or higher temperatures may result in reduced cDNA yields.

20. Generated cDNA may also be stored diluted (i.e., 1:5 vol:vol).
21. Primer sequences can be derived from scientific literature or designed by means of dedicated informatic tools. We employ the "Primer Express" software to design primer sequences according to ABI recommendations.
22. Volume assay should be adjusted accordingly to the available real-time PCR instrument.
23. The optimal dilution of cDNAs to obtain a PCR product within the linear phase of the amplification should be established in preliminary experiments.

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Chapter 8

Laser Capture Microdissection and Isolation of High-Quality RNA from Frozen Endometrial Tissue

Michele Cummings, Georgia Mappa, and Nicolas M. Orsi

Abstract

Laser capture microdissection (LCM) allows expression profiling of specific cell populations within tissues. However, isolation of high-quality RNA from laser capture microdissected frozen tissue is beset by problems arising from intrinsic tissue RNase activity. Herein, we describe an optimized staining/LCM/RNA extraction protocol developed for the isolation of epithelial RNA from frozen tissue sections using human endometrial cancer as a model tissue. This method combines excellent, reproducible visualization of tissue morphology with the isolation of high-integrity RNA suitable for downstream applications such as expression microarray analysis. We present quantitative and qualitative RNA data obtained from >200 endometrial epithelial samples (normal, hyperplastic, and cancerous), where 92% of samples had RIN values of 7 and above and highlight common pitfalls faced by investigators. This method should also be broadly applicable to a range of other tissue types.

Key words Frozen tissue, Laser capture microdissection, Cresyl violet, RNA integrity, RIN, Endometrium, Epithelium

1 Introduction

Laser capture microdissection enables the visualization and isolation of homogeneous cell populations from tissue sections for subsequent downstream molecular analyses. However, isolation of high-quality RNA suitable for whole genome expression profiling via microarray [1] or RNA sequencing [2] technologies presents a particular challenge due to the ubiquity of RNases. These enzymes are present in varying amounts in different tissues [3] and degrade RNA during ex vivo autolytic processes. Most problematic for LCM is the fact that RNases are released from cells upon freeze-thawing of tissue and cause rapid RNA degradation. Thus, the necessity for the adequate visualization of tissue morphology for accurate LCM must be balanced against the need to inactivate tissue RNases. Unfortunately, since storage in tissue RNA stabilizers (e.g., RNAlater) fails to maintain adequate tissue morphology

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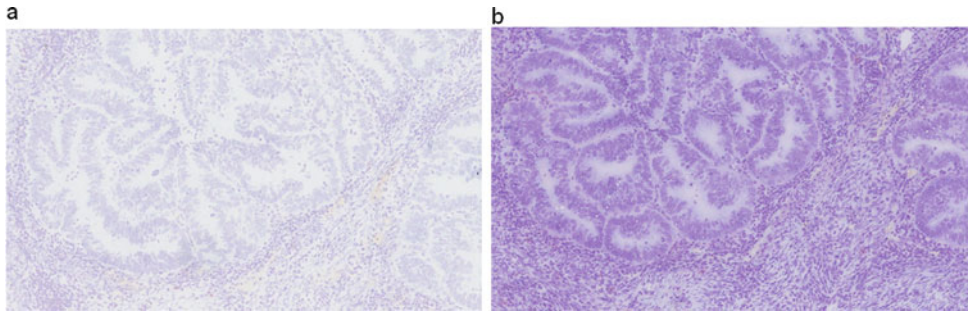


Fig. 1 Duplicate 8 μm frozen sections of endometrial adenocarcinoma were mounted on glass slides and stained using the protocol described in Subheading 3.3. Alcohol-based cresyl violet stain was buffered with 20 mM (final concentration) Tris–HCl pH 7.0 (**a**), and pH 8.0 (**b**)

[4], one is inevitably tied to using fresh frozen tissue. While commercially developed stains developed for LCM/RNA isolation (e.g., HistoGene) work well with certain tissues such as brain [5], we [6] and others [7, 8] have found that the use of this stain is associated with significant RNA degradation. In order to minimize RNase activation during tissue staining and LCM, various approaches have been adopted, such as the inclusion of RNase inhibitors in aqueous stains [8], or the use of alcohol-based stains incorporating cresyl violet [9] which limit tissue hydration and hence RNase activation. In our hands, alcohol-based cresyl violet gave the best results in terms of tissue morphology and allowed the isolation of high-integrity RNA from LCM of epithelium [6]. We also found that both the staining intensity and reproducibility achieved using alcohol-based cresyl violet solutions were improved by adjusting the pH of the stain to pH 8.0 prior to use (Fig. 1). Our protocol also incorporates the addition of an appropriate RNase protection reagent immediately after RNA isolation in order to inactivate any residual co-purified tissue RNase.

2 Materials

2.1 Standard Equipment/Materials

1. Use a commercial RNase-free water (not DEPC-treated) and ACS grade absolute ethanol to prepare all fixing/staining solutions. Glassware/non-disposable plasticware should be sprayed with RNase-ZAP (Fisher Scientific, Loughborough, UK), rinsed thoroughly in Milli-Q water and allowed to dry prior to use.
2. Anhydrous ethanol (for the final slide dehydration steps) is made by adding 15 g of 3-Å molecular sieve beads to 500 ml ethanol (prepare this the day before so as to allow the beads to settle fully and store bottle tightly closed thereafter).

3. Alcohol-based cresyl violet stock is made by dissolving 1 g cresyl violet acetate per 100 ml 75% (v/v) ethanol overnight on a magnetic stirrer and protected from light. The solution is then filtered through Whatman Grade one filter paper and stored at room temperature, again protected from light (this keeps for months). Just prior to staining (*see* **Note 1**), take the required amount of cresyl violet stock and adjust the pH by adding 1 M Tris–HCl (pH 8.0) so that the final concentration of Tris is 20 mM (i.e., add 20 μl Tris–HCl per 1 ml cresyl violet stock).

2.2 Specialist Equipment/Materials

1. Cryostat (CM3050S; Leica Microsystems; Wetzlar, Germany).
2. Laser Capture Microscope (PALM MicroBeam UV; Carl Zeiss, Herts., UK).
3. Polyethylene naphthalate (PEN) membrane-coated glass slides (Carl Zeiss or Arcturus), adhesive capped collection tubes (Carl Zeiss).
4. RNeasy plus micro kit (Qiagen, Crawley, UK).
5. RNase inhibitor: RNAsecure or SUPERase-In (Ambion; Thermo Fisher Scientific)
6. Bioanalyzer 2100 (Agilent technologies).
7. RNA 6000 Nano kit (Agilent Technologies).
8. SpeedVac with a cold trap (Savant SC110 or similar; if required).

3 Methods

3.1 Tissue Collection and Storage

Tissue should be transferred to ice and snap frozen as quickly as possible in order to minimize autolytic RNA degradation or changes in RNA expression profiles due to warm ischemia [10].

1. For the human endometrial samples used in this study (ethical approval Ref: 05/Q1107/41), hysterectomy specimens were immediately collected from theater and endometrial tissue sampled by a histopathologist and placed into labeled tubes. Samples were transported to the laboratory on wet ice, wrapped in foil and snap frozen in liquid nitrogen (*see* **Note 2**) within 1 h.
2. Tissues were stored long term at −80 °C in tightly sealed tubes to prevent desiccation. Alternatively, tissue can be embedded in optimal cutting compound (OCT) if preferred (*see* **Note 2**). However, a modification of the staining procedure is required to remove residual OCT from tissue sections (*see* **Note 3**). We have used this latter approach to obtain high-integrity RNA from laser capture microdissected murine endometrial stroma and epithelia [11].

3.2 Cryosectioning

Tissue should only be handled on dry ice with previously cooled forceps/tweezers. At no point should tissue be allowed to thaw even slightly. Either an anti-roll plate or a natural fibre artist's paintbrush can be used to stop sections rolling. Clean the cryostat stage, brushes, tweezers, and anti-roll plate with ethanol before use. Change the microtome blade before use and move along to an unused area when sectioning a new specimen. Make sure the handwheel of the cryostat is locked at all times except when sectioning.

1. Set the chamber and chuck temperature in the cryostat to −16 °C (this is ideal for endometrial tissue but may need optimizing for different tissues). Once the desired temperature is reached, place the tissue (wrapped in foil) in the cryostat chamber and allow to equilibrate for 15 min–1 h depending upon the size of the specimen.
2. Set the section thickness on the cryostat to 8 or 12 μm (*see* **Note 4**). Set the trim thickness to 30 μm.
3. Remove the tissue from the foil with tweezers and mount on the chuck (prechilled inside the cryostat) using RNase-free water which will freeze it in place (*see* **Note 5**). Alternatively, use OCT to mount OCT embedded tissue.
4. Secure the chuck in the chuck stage.
5. Unlock the handwheel and check the distance of the specimen from the blade and bring the specimen very close to the blade.
6. Set to "trim" and use the handwheel to trim tissue until a suitable "face" is reached. Be careful not to over-trim so as to minimize tissue wastage.
7. To take sections, brush away tissue trimmings, turn the trim function off, and cut a few sections (the first few will typically be thicker than 8 μm). If not using an anti-roll plate, cut slowly and tease the tissue slowly away from the blade using a paintbrush to hold the edge.
8. Mount sections on either uncharged glass slides or PEN slides (for LCM) stored at room temperature. Depending on the section size, more than one section may be mounted on a PEN slide, which is more economical. Arrange tissue section(s) on the cryostat stage using a paintbrush. Label slide, hold at the labeled end, and rest the opposite edge on the stage below the tissue section. Pivot to lower the slide quickly and gently (membrane side down) onto the section(s); the section(s) will adhere to the slide as it thaws very briefly. Quickly place the slide face-up on the cryobar within the cryostat and allow to freeze for a few seconds. Wrap the slide(s) loosely in foil within the cryostat chamber and transfer quickly to dry ice for transport to the staining bench.

9. To check that a suitable area of tissue is selected prior to mounting on PEN slides, sections can be mounted on uncharged glass slides and visualized by cresyl violet quick stain (*see* **Note 6**). Glass slides of tissue sections should also be prepared for standard hematoxylin and eosin (H&E) staining as a permanent record and for histopathological assessment since the nature of the tissue (e.g., cancer vs. background normal tissue) can be presumptive based on its macroscopic assessment at the time of collection.
10. Once cryosectioning is complete, carefully remove the mounted tissue from the chuck using a razor blade to cut through the ice and transfer the tissue quickly to dry ice prior to storage.

3.3 Staining for LCM

This protocol is for non-OCT embedded tissue sections; for OCT embedded sections the protocol is modified slightly (*see* **Note 3**).

1. Fill coplin jars as follows: A, D (95% ethanol), B, C (75% ethanol), E, F, G (anhydrous ethanol)—*see* Subheading 2.1.
2. Calculate the amount of cresyl violet stain required for the experiment (based on needing 250 μl per slide) and adjust the pH to 8.0 with Tris–HCl (*see* Subheading 2.1).
3. Cryosectioned tissue slides should be stored face up on dry ice, wrapped loosely in foil, until use. Slides should be stained sequentially rather than in batches and up to 30 slides can be stained before changing the ethanol solutions. Dab off excess liquid from the slide in between each step by placing the edge of the slide on absorbent tissue paper. Stain as follows:
   (a) Immerse slide up to frosted edge in jar A (95% ethanol) for 30 s.
   (b) Immerse slide as above in jar B (75% ethanol) for 30 s.
   (c) Lay slide flat and face up on absorbent tissue. Using a hydrophobic barrier pen, draw two lines either side of the PEN membrane parallel to the frosted edge of the slide.
   (d) Pipette 250 μl cresyl violet stain (prepared in ii) evenly over the tissue. Wait 30 s and then remove excess stain by dabbing the long edge of the slide on a tissue.
   (e) Immerse the slide in jar C (75% ethanol) for 30 s.
   (f) Immerse the slide in jar D (95% ethanol) for 30 s.
   (g) Immerse the slide in jar E (anhydrous ethanol) for 30 s.
   (h) Immerse the slide in jar F (anhydrous ethanol) for 30 s.
   (i) Immerse the slide in jar G (anhydrous ethanol) for 5 min (one can commence staining the next slide at this point).

(j) Blot off excess ethanol from the edge of the slide and lay face side up to air-dry. Either proceed directly to LCM or store the slide at −80 °C as described in Subheading 3.4.

3.4 Slide Storage

Place the slides individually in 50 ml Falcon tubes containing ~5 ml pre-dried silica desiccant beads. Close tightly, seal with Parafilm, and store at −80 °C. Slides can be stored at least 2 months in this way without any deterioration in RNA integrity. *Important:* always allow tubes to equilibrate to room temperature completely before opening in order to prevent condensation forming on the slides, which would activate tissue RNases.

3.5 Laser Capture Microdissection

Laser capture microdissection was carried out using a PALM MicroBeam UV LCM microscope, using the Palm Robo software, version 3.2. Gloves must be worn throughout.

1. Before starting, check the relative humidity level in the laboratory, which should ideally be 45% or less (*see* **Note 7**).
2. Remove the selected slide from the −80 °C freezer (Subheading 3.4), and allow it to equilibrate to room temperature *before* removing it from the storage tube.
3. Switch on the LCM microscope and allow to warm up (the indicator light turns from orange to green when the laser can be switched on). Open the Palm Robo software and load the PEN slide and the adhesive-capped collection tube as per the manufacturer's instructions.
4. Ensure energy, focus, and laser alignment are suitable on an area of PEN membrane free of tissue: Use the freehand draw tool to draw a line, select "cut" and realign laser with the crosshairs on the screen if necessary using the "Position UV laser" option.
5. Position the diffusor above the slide to enable better tissue visualization and draw around selected areas (at 10–20× magnification).
6. When ready to cut, position the collection tube above the slide; select the drawn areas (which can be viewed on the "elements list") and harvest using the Robo LPC function with your pre-optimized settings (*see* **Notes 8** and **9**).
7. Once enough material has been collected (or if all the available material on a particular slide has been collected), add an appropriate volume (*see* **Note 10**) of RLT plus buffer (Qiagen RNeasy plus microkit) containing freshly added β-mercaptoethanol to the collection tube. Vortex thoroughly to solubilize the LCM material (invert several times to ensure removal of material from the lid) and either extract immediately or store at −80 °C until ready to perform RNA isolation (*see* **Note 11**).

3.6 RNA Isolation, Quality Control, and Quantitation

For RNA extraction and handling, use a dedicated set of equipment (pipettes and microfuge), RNase-free plasticware, and barrier tips throughout. RNA isolation from LCM material is carried out on the LCM lysate using the Qiagen RNeasy plus micro kit (*see* **Note 12**). Manufacturer's instructions are followed but with the following modifications:

1. An additional 80% ethanol wash step is performed to remove any traces of guanidinium salts.
2. An RNA protection reagent (RNAsecure; *see* **Note 13**) is added to the RNA immediately on purification as follows: RNAsecure reagent (25×; 0.5 μl) is added to the bottom of the eluate collection tube prior to eluting RNA from the column with 14 μl RNAse-free water (12 μl RNA is eluted).
3. Samples are then incubated for 10 min at 60 °C to inactivate RNases and subsequently evaporated to dryness using a SpeedVac with a cold trap at ambient temperature and resuspended in 5 μl RNase-free water.
4. A sample (1 μl) is run on a RNA 6000 Nano LabChip (*see* **Notes 14 and 15**) for both RNA quantitation and RIN measurements (Fig. 2) and the remainder of the samples stored at −80 °C.

For comparison, RNA was extracted from whole frozen sections (8 μm thick, about 1–3 cm^2 surface area) of endometrial cancer specimens. Tissue was mounted on glass slides and stained with alcohol-based cresyl violet as described for the LCM specimens. Tissue was then scraped from the slide into RLT plus buffer (Qiagen) pipetted onto the section, vortexed thoroughly and passed through a Qiashredder column (Qiagen) before extracting with RNeasy plus micro kit as described (omitting the SpeedVac concentration step).

Results of 203 separate LCM/RNA extractions from normal, hyperplastic, and cancerous human endometrial epithelia from a total of 131 study participants are shown in Fig. 3. There was no significant difference in RIN values between normal, hyperplastic, or cancerous LCM RNA samples (Kruskall-Wallis test) but a significant inverse correlation (Pearson's product moment correlation $P < 0.001$) between time taken to LCM and RIN value was identified, although the estimated effect of this was modest (0.24 RIN unit decrease per hour, a figure that agrees well with our previous experimentally derived estimate of 0.1–0.3 RIN unit per hour) [6]. Taken as a whole, the vast majority of LCM RNA samples (91.6%) had RIN values of 7 and above, and only 1.5% of samples had RIN values <5. No significant correlation was observed between RIN values and time taken from collection to freezing (data not shown), although this variable was kept to a minimum. No significant differences were observed in RNA yields

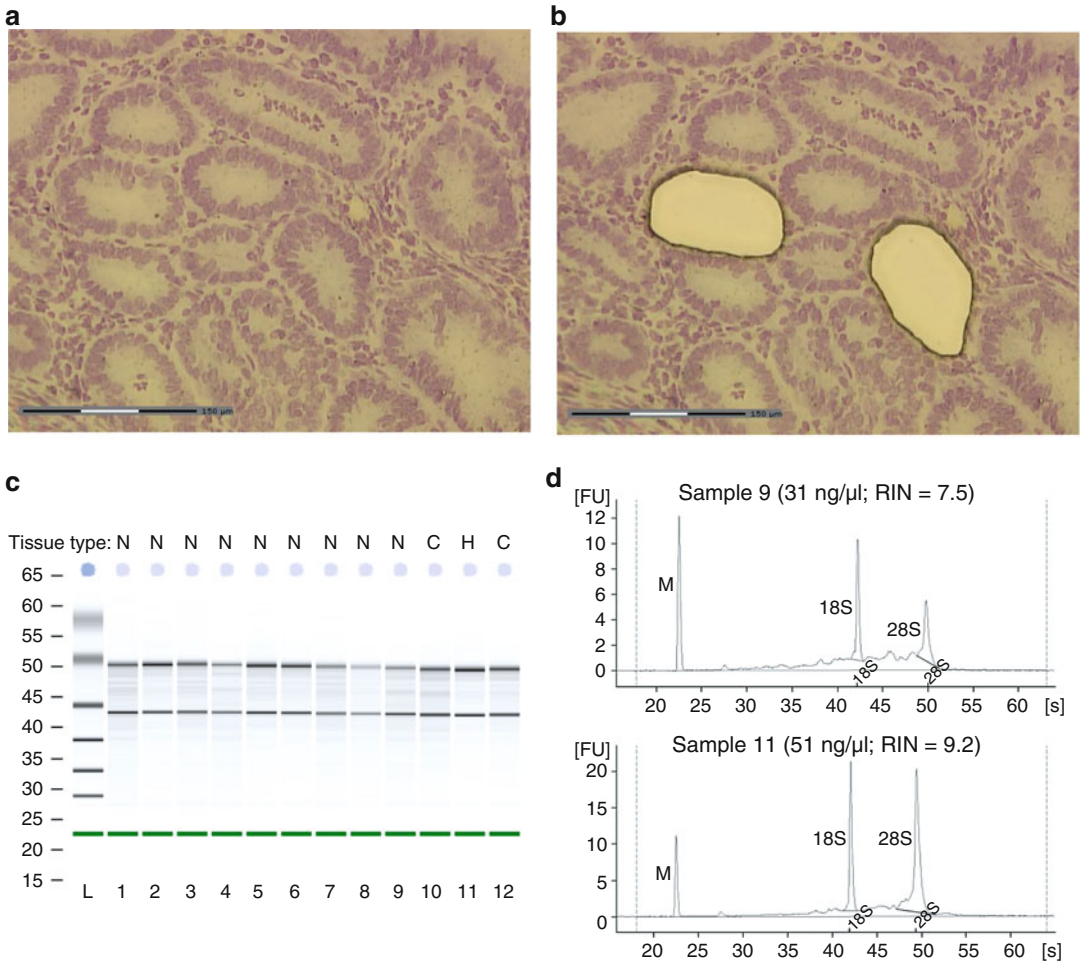
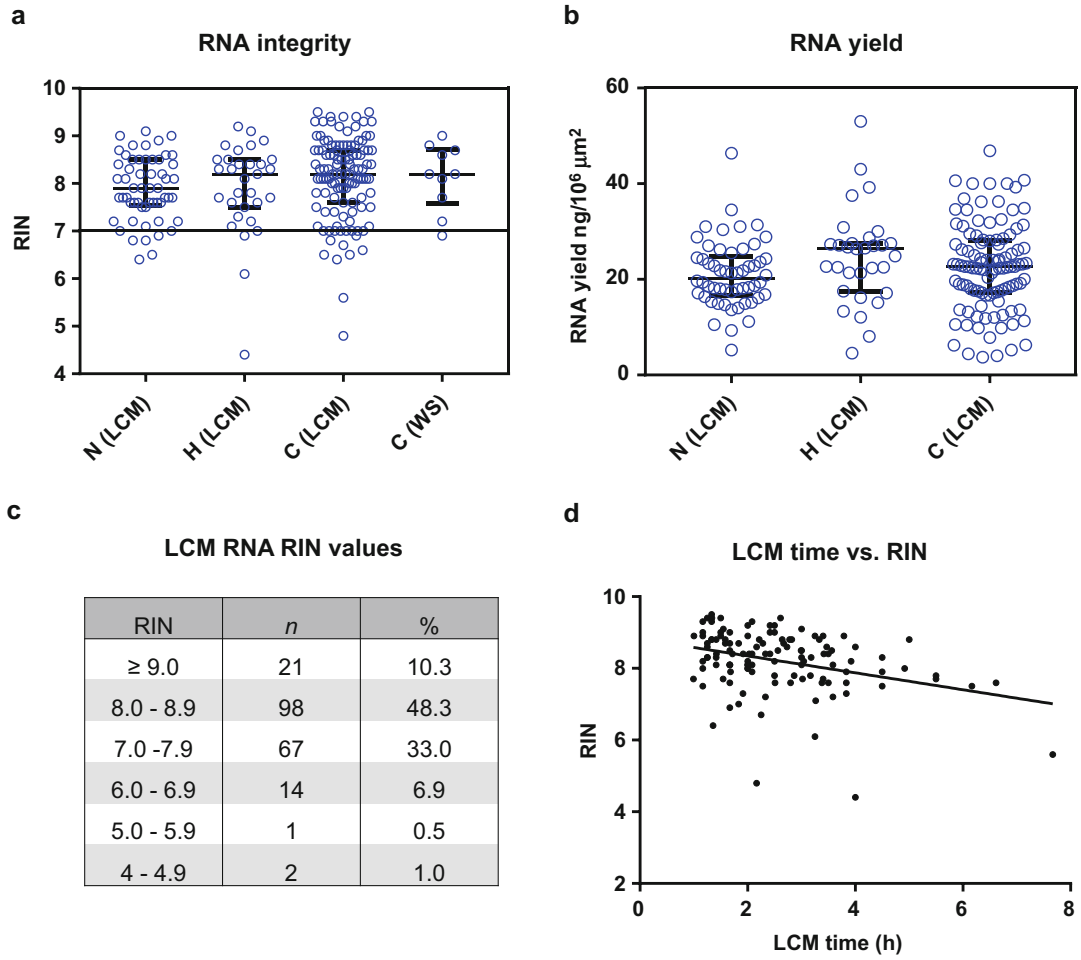


Fig. 2 (**a**, **b**) An endometrial adenocarcinoma frozen tissue section mounted on a PEN membrane slide and stained with alcohol-based cresyl violet (buffered to pH 8.0) according to the protocol described in Subheading 3.3. The section is depicted before (**a**) and after (**b**) LCM of epithelial nests. (**c**) A typical Bioanalyzer pseudogel image obtained after running LCM endometrial epithelial RNA on an RNA 6000 Nano LabChip (N, H, C correspond to RNA extracted from normal, hyperplastic, and cancerous endometrial epithelia, respectively). Examples of Bioanalyzer traces from samples 9 and 11 with corresponding RIN and RNA concentrations are depicted in (**d**); 28S and 18S ribosomal RNA peaks are indicated, as well as the internal marker (M)

between the different types of epithelia (normal, hyperplasia, cancer). The median RNA yield for all the samples was 23 ng/10^6 µm^2 (interquartile range 17–27).

4 Notes

1. Adjusting the pH of the cresyl violet stock with Tris–HCl pH 8.0 was found to give more intense and reproducible tissue staining. However, it does cause slight precipitation of cresyl



RIN	*n*	%
≥ 9.0	21	10.3
8.0 - 8.9	98	48.3
7.0 -7.9	67	33.0
6.0 - 6.9	14	6.9
5.0 - 5.9	1	0.5
4 - 4.9	2	1.0

Fig. 3 (**a**) RIN values (median and interquartile range) of RNA extracted from normal (N), hyperplastic (H), and cancerous (C) human endometrial epithelia ($n = 203$) isolated by LCM from 8 μm thick frozen sections using the optimized protocol described herein. RIN values for RNA extracted from whole frozen sections of endometrial cancer tissues from different patients (WS; $n = 10$) are also shown for comparison. RNA yields per unit surface area (median and interquartile range) obtained from the same LCM samples are shown in (**b**). RIN values for all LCM RNA samples are summarized as a table in (**c**) and the relationship between LCM time and RNA quality is shown in graph (**d**), where the slope of the linear regression curve is -0.24 ± 0.06 RIN units/h; the Pearson correlation coefficient of LCM time vs RIN is -0.34, $P < 0.001$

violet, although this occurs slowly and the stain is good to use over the course of an hour. For this reason, the required amount of cresyl violet stock for the staining experiment should be pH-adjusted just prior to starting.

2. Snap freezing in isopentane slurry cooled on liquid nitrogen is an alternative freezing method and can give better preservation of morphology in some tissues since the tissue freezes more quickly as it is not insulated by a layer of nitrogen gas. This method is compatible with nucleic acid isolation but may not

be compatible with other potential applications such as lipid analysis. Similarly, the use of OCT enables greater ease in frozen sectioning but OCT may interfere with potential downstream applications, although it is compatible with RNA isolation (*see* **Note 3**). Snap freezing directly in liquid nitrogen without OCT affords the greatest versatility for downstream analyses (important for clinical samples), and in our experience endometrial tissue morphology is largely unaffected using this approach.

3. To remove residual OCT the staining protocol described in Subheading 3.3 is modified by the inclusion of an additional 30 s incubation in 50% ethanol both immediately before and immediately after the cresyl violet staining step.
4. Sections of 12 μm thickness are advantageous compared to 8 μm since more material is harvested per unit area during LCM. However, in our experience, cutting 8 μm sections allows better consistency in section quality.
5. Mounting tissue onto the cryostat chuck using water requires good timing. The trick is to place a small blob of water (50–150 μl, depending on the size of tissue) on the chuck using a plastic pastette, allow the water to freeze just enough (so it starts to become slightly opaque) before placing the tissue on the water mound, where it will adhere as it freezes. If the water freezes for too long prior to placing the tissue it will not adhere properly. However, not allowing the water to freeze sufficiently will result in the tissue sitting in a puddle of water which will risk tissue thawing and seriously compromise RNA integrity.
6. To visualize tissue sections quickly while sectioning, we found it convenient to use a quick cresyl violet staining protocol: remove the glass slide from dry ice and immerse in 95% ethanol for 30 s to fix, blot and stain with buffered cresyl violet stain (*see* Subheading 2.1) for 30 s, blot and immerse in 2× absolute ethanol (not anhydrous) washes for 30 s each. Proceed to collecting sections for mounting on PEN slides for LCM and glass slides for H&E if satisfied with the area of tissue selected.
7. Other researchers [5] have noted that humidity levels can affect RNA quality from LCM specimens, presumably due to tissue rehydration and activation of RNases. As a precautionary measure, we used a portable dehumidifier (MEACO DD8L Junior) to avoid humidity levels exceeding 45%.
8. Optimized cutting settings for our purposes were at 10× magnification, set to a speed of 55% maximum with cutting and LPC (laser pulse catapult) energies of 49% and 90% of maximum, respectively, although the cutting energy required did

need to be increased for some of the normal endometrial specimens.

9. Although tempting, avoid harvesting too many areas at once as the collection cap becomes "saturated" with pieces of tissue. Periodically inspect the collection tube cap at 5× magnification and adjust its x and y coordinates to align an empty area above the slide. Pieces that do not adhere can be identified on the slide at 5× magnification and catapulted onto the cap using the LPC function.
10. In this study, ~4–8 × 10^6 μm^2 (preferably ~6 μm^2) of LCM epithelia was harvested to obtain the yields stated. If necessary, material from different slides can be harvested separately and combined before extracting, ensuring the final volume of RLT plus buffer lysate is 350 μl.
11. LCM tissue lysates in RLT plus buffer with β-mercaptothanol can be stored for months at −80 °C before extracting with no apparent loss of RNA integrity.
12. We used the standard manufacturer's protocol which purifies RNA species >200 nucleotides. A modified manufacturer's protocol is available for the co-purification of small RNA species if desired, although we have not tested this. We have not compared this kit with others but suggest that approaches using phenol/chloroform be avoided for limited material owing to RNA losses at the aqueous/organic interface.
13. We found RNAsecure to be an ideal RNase inhibitor as it irreversibly inactivates any residual tissue RNase in purified RNA from tissue samples. RNA preserved in this way (25 ng) was used successfully in microarray hybridization experiments using Agilent SurePrint G3 Human GE 8 × 60 k microarrays, where cRNA probes generated using the Agilent Low Input Quick Amp One Color labeling kit had specific activities and yields that met the recommended levels for array hybridization and subsequent microarray data passed all Agilent QC metrics. However, we recommend that users check compatibility of RNAsecure with their downstream applications. Specifically, RNAsecure is active above 45 °C and may inhibit certain enzymes during incubations above this temperature. As an alternative, SUPERase-In (a broad spectrum protein-based RNase inhibitor) may be added to purified RNA, although in this case RNase inactivation is potentially reversible.
14. Note that RNA concentration was necessary for the purposes of our downstream analysis and to bring the RNA within the measurable range of the RNA 6000 Nano LabChip. The Agilent RNA 6000 Pico LabChip could be used as an alternative, depending on RNA concentration requirements. As Pico

LabChips are very sensitive to the presence of ions, check whether runs are affected by any RNA protectants used.

15. Note also that addition of either RNAsecure or SUPERase-In renders UV spectrophotometric readings (nanodrop) unreliable but does not affect quantitation methods based on fluorescence (e.g., Bioanalyzer concentration readings).

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Chapter 9

Laser Microdissection for Human Papillomavirus (HPV) Genotyping Attribution and Methylation Pattern Analyses of Squamous Intraepithelial Lesions

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Abstract

Human papillomavirus (HPV) is a nearly ubiquitous infectious organism. It is estimated that 80% of sexually active adults will be exposed to anogenital HPVs in their lifetime, and detection of multiple genotypes in an anogenital sample is common. Detection and genotyping of HPV is usually performed by DNA testing, and less frequently by mRNA testing. HPV genotype testing and characterization of DNA methylation patterns of HPV-related lesions can provide important biological, epidemiological, and potentially relevant clinical information in individuals and populations. The use of laser capture microdissection to isolate cells within a specific lesion allows for very precise molecular characterization and hence causal attribution. This chapter describes detailed protocols for the capture of lesion-specific tissue from formalin-fixed, paraffin-embedded (FFPE) biopsy tissue, and downstream DNA testing for lesion-specific HPV genotype and their methylation patterns.

Key words Laser capture microdissection, Human papillomavirus, Genotyping, Methylation sequencing, FFPE

1 Introduction

Human papillomavirus (HPV) is the most common sexually-transmitted infectious organism, with an estimated 80% of sexually active adults being exposed in their lifetime [1]. Detection of multiple genotypes in an anogenital sample is common, and therefore attributing a particular genotype to a lesion or cancer can be difficult. Several mathematical algorithms have been developed to estimate the most likely causative genotype in such samples [2]; however, the accuracy of these remains untested and they are more likely to be accurate at the population-wide level, than at the individual level. The use of laser capture microdissection (LCM). of anogenital biopsy specimens has been consistently demonstrated to be a useful method for resolving a single genotype per individual

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lesion, even in biopsies that as a whole test positive for multiple genotypes [3–7].

As the drive to prevent HPV-related cancer continues in an era where vaccine-related HPV infections and associated lesions are in decline, and consequently the sensitivity of cervical cytology as a primary screen for lesions is also decreasing, more sensitive subjective markers such as HPV DNA are being introduced [8]. Being so common, however, HPV DNA detection is not sufficiently specific to indicate disease, therefore increasingly DNA methylation patterns are being investigated as potential biomarkers of disease progression and oncogenic potential, possibly even as therapeutic targets [9–11]. It is also known that methylation patterns differ between lesion-associated cells and adjacent normal tissue, as well as between different layers within the same lesion [12]. LCM therefore presents a powerful tool for precise characterization of methylation patterns in HPV-related lesions.

Anogenital biopsies can be very small, resulting in low-quantity DNA samples. Additionally, formalin fixation and subsequent deparaffinization results in fragmented DNA [13]. Therefore, the methodologies developed are very sensitive, both for HPV DNA detection and for amplification and sequencing for methylation analysis. As a consequence, extra precautions have been indicated to minimize the risk of cross-contamination, particularly with incidental HPV DNA. With these points in mind, the following protocols have therefore been developed for formalin-fixed, paraffin-embedded (FFPE) tissue.

2 Materials

The choice of immunohistochemical staining method is up to the discretion of the research team, depending on what is available to them. Manual or automated staining are both acceptable, and sectioning and staining can also be outsourced to an accredited pathology laboratory. Suggestions for an automated system are included in this protocol. For manual staining, please refer to a reputable immunohistochemistry guide [14].

There are several types of LCM systems, for example the single-laser PALM MicroBeam (Zeiss Microscopy) and dual laser Arcturus (Applied Biosystems) systems. The reagent requirements for the Arcturus system are described here. Similarly, there are several options for HPV DNA genotyping kits, methylation-related reagents such as bisulfite conversion kits, and methylation-specific sequencing or detection systems. Pyrosequencing is a sensitive method that allows quantification of methylation at multiple individual CpG sites within a target sequence. Specific protocols have been provided for selected kits and systems which have proven to work well on LCM-isolated HPV-related lesions.

2.1 Tissue Sectioning

1. Formalin-fixed, paraffin-embedded (FFPE) tissue blocks prepared in the standard way.
2. Polyethylene naphthalate (PEN) membrane slides.
3. Microtome.
4. Disposable microtome blades.
5. Para-Kleaner.
6. Sterile water.
7. Water bath.
8. Xylene (100%).
9. Ethanol (95 and 100%).
10. Adhesive immunohistochemistry slides.
11. Leica Autostainer and Coverslipper (Leica Microsystems).
12. Amber Alcoholic Eosin.
13. Gills No. 2 hematoxylin.
14. Scott's Tap Water Substitute.
15. Acid alcohol 0.25%: 9.975 L of 70% ethanol, 25 ml of 25% hydrochloric acid (*see* **Note 1**).
16. Ventana Benchmark Ultra instrument (Ventana Medical Systems, Inc., Oro Valley AZ).
17. CINtec anti-p16 (Roche, Tucson AZ).
18. Aperio digital scanner and ImageScope software (Leica Microsystems).

2.2 Laser Microdissection

1. Xylene (100%) (*see* **Note 2**).
2. Ethanol (100%).
3. 50 ml sterile polypropylene centrifuge tubes.
4. Centrifuge tube rack.
5. Chemical fume cupboard suitable for handling solvents.
6. Absorbent paper towelling.
7. Disposable tweezers or forceps.
8. Hybridization oven or incubator.
9. Silica gel dessicant beads.
10. Arcturus Capsure Macro LCM Caps (Thermo Fisher Scientific).
11. 0.5 ml microcentrifuge or PCR tubes.
12. Arcturus Veritas LCM system (Thermo Fisher Scientific).

2.3 DNA Extraction and Molecular Genotyping

1. PCR cabinet or Class II Biological Safety Cabinet.
2. Microcentrifuge tube opener.

3. Arcturus PicoPure DNA Extraction Kit (Thermo Fisher Scientific).
4. Microcentrifuge.
5. Sterile 1.5 ml microfuge tubes if required.
6. Heat block.
7. SensiFast Probe No-ROX mix (Bioline, London, UK).
8. Human genomic DNA from human blood.
9. 110 base-pair beta-globin primer and probe set [4].
10. LightCycler 480-compatible 96-well PCR microplate.
11. LightCycler 480 Real-time quantitative PCR system (Roche).
12. RHA kit HPV SPF10-LiPA25, version 1 (Labo Bio-medical Products BV, Rijswijk, The Netherlands).
13. Auto LiPA Autoblotter (Innogenetics N.V, Ghent, Belgium), for automated genotyping.
14. Water bath and rocking platform, for manual genotyping.
15. DNA ELISA kit HPV SPF10, version 1 (Labo Bio-medical).
16. SPF+ detection strips (Labo Bio-medical).
17. Genotype-specific qPCR primer and probe sets [4, 15].

2.4 Bisulfite Conversion

1. Control HPV16-positive cervical cell line CaSki (ATCC CRL1550) or SiHa (ATCC HTB35) (American Type Culture Collection (ATCC), Manassas VA).
2. Methylamp DNA modification Kit (Epigentek, Brooklyn NY).
3. Heat block.
4. Vortex mixer.
5. Microcentrifuge (up to 14,000 rpm/18,000 × *g*).
6. Sterile 1.5 ml microfuge tubes.
7. Ethanol (100%).
8. High purity water.

2.5 DNA Purification after Bisulphite Conversion

1. QIAamp DNA Mini Kit (Qiagen, Hilden, Germany).
2. Sterile 1.5 ml microfuge tubes.
3. Heat block.
4. Vortex mixer.
5. Microcentrifuge (up to 14,000 rpm/18,000 × *g*).

2.6 Agarose Gel Electrophoresis

1. Buffer 1× tris acetate-EDTA (TAE) (Sigma-Aldrich, St. Louis MO).
2. Agarose I (Amresco, Solon OH).

3. 6× DNA loading buffer: 0.25% Bromophenol Blue, 0.25% xylene cyanol, 30% Glycerol.
4. GelGreen Nucleic Acid Gel Stain, 10,000× (Biotium, Fremont CA).
5. DNA molecular weight markers: EasyLadder I or HyperLadder 100 bp (Bioline).
6. Horizontal gel electrophoresis apparatus and DC power supply.

2.7 PCR Amplification for Methylation Analysis

1. Primers for PCR amplification and pyrosequencing, including biotinylated primers [15, 16].
2. HotStart Taq master mix (Qiagen).
3. Nuclease-free water.
4. 96-well PCR microplate.
5. Thermal cycler.

2.8 Methylation Analysis Using Pyrosequencing

1. PyroMark Q24 Advanced System (Qiagen).
2. Streptavidin Sepharose High Performance 34 μm beads, 5 ml (GE Healthcare, Little Chalfont, UK).
3. PyroMark Q24 Advanced Reagents (Qiagen).
4. 5′-biotinylated PCR product (5–20 μL per reaction).
5. PCR plate.
6. Adhesive foil.
7. Plate mixer for immobilization to beads.
8. Heating block.
9. PyroMark Q24 Plate (Qiagen).
10. PyroMark Q24 Cartridge (Qiagen).
11. Pyrosequencing primers [15, 16].
12. High-purity water (Milli-Q 18.2 MΩ cm or equivalent).
13. Ethanol (70%).
14. PyroMark Denaturation Solution (Qiagen).
15. PyroMark Wash Buffer concentrate (Qiagen).
16. Pipettes and sterile pipette tips with filters.

3 Methods

The following protocol describes all major procedures required to produce lesion-specific HPV genotype and methylation data from HPV-related squamous lesions. The protocol describes histological processing of formalin-fixed, paraffin-embedded (FFPE) biopsy specimens for annotation and LCM to minimize cross-

contamination with incident HPV DNA and to ensure accurate identification of histological lesions. DNA extraction of microdissected tissue and testing of DNA integrity are described, followed by a sensitive reverse line probe HPV genotyping method, including detection of rare mucosal genotypes, and a genotype-specific qPCR targeting the E6 reading frame to rule out false negative results caused by HPV genome integration at the L1 open reading frame. Detailed instructions for bisulfite conversion and DNA purification are followed by a method for sensitive amplification of CpG-rich regions of the HPV16 viral upstream regulatory region, and the protocol concludes with steps for methylation-specific pyrosequencing. It will be obvious to the reader that the methylation sequence targets we describe will only be useful for lesions that test positive for HPV16; we encourage development and testing of other nested primer sets for alternative targets (viral and host) using the principles described.

3.1 Tissue Sectioning and Annotation of Lesions

Stringent measures must be taken to minimize cross-contamination of samples. This is because biopsy samples may contain more than one genotype, the presence of HPV DNA from other sources is also very common, and HPV DNA assays for FFPE tissue are very sensitive. Prevention of contamination is most important during and between tissue sectioning. PCR-sterile reagents and equipment should be used for each biopsy, and where possible each biopsy should be oriented such that the microtome blade passes through the superficial mucosal layer last.

Prior to performing LCM, lesion location and grade should be identified by a histopathologist. Where a histopathologist is not available, positive block staining for p16-ink4A may be used as a surrogate for high-grade lesions; however, a proportion of low-grade lesions will test positive for p16 [17]. Conversely, p16-negative HPV-related cancers have also been observed [18]. It is noteworthy that positive p16-staining is not useful for discriminating between HSIL grades 2 and 3, or between HSIL and squamous cell carcinoma. It should also be noted that lesion grade may change (usually downgraded) between the original diagnosis and review of new sections for LCM. It is up to individual research teams to decide which diagnosis to use in their analysis; however, in most cases, it is recommended that the review diagnosis be used.

1. Prior to sectioning each biopsy, clean the microtome surface with xylene or ParaKleaner followed by ethanol, and change to a new sterile disposable microtome blade (also cleaned with ethanol), and prepare a new water bath for floating sections to mount onto slides.
2. Process each biopsy for histological analysis and LCM using a sandwich sectioning method (modified from [4, 5]). Where

possible without resetting the blocks, cut biopsies from the basal side toward the epithelial/superficial side to minimize carryover of potentially colonized surface viral DNA on lesional tissue. Cut sections 1, 2 and 4 to 3 μm thickness and mount onto adhesive glass immunohistochemistry slides. Cut section 3 to 9 μm thickness and place onto a PEN membrane glass slide. Allow mounted sections to dry.

3. Stain the first and last section with hematoxylin and eosin (H&E) using the Leica Autostainer and Coverslipper (*see* **Note 3**).
4. Stain the second section with CINtec anti-p16 and counterstain with Gills No. 2 hematoxylin on the Ventana Benchmark Ultra instrument (*see* **Note 3**).
5. Scan the three stained slides on the Aperio digital scanner at 20 × magnification.
6. Send the glass slides and digitised images to an anogenital histopathologist for review. Using H&E morphology, supported by p16 staining as indicated according to international criteria [17], areas of interest for LCM and associated disease grade are to be indicated on the digitised slide image using the drawing tool in Aperio ImageScope (Fig. 1).

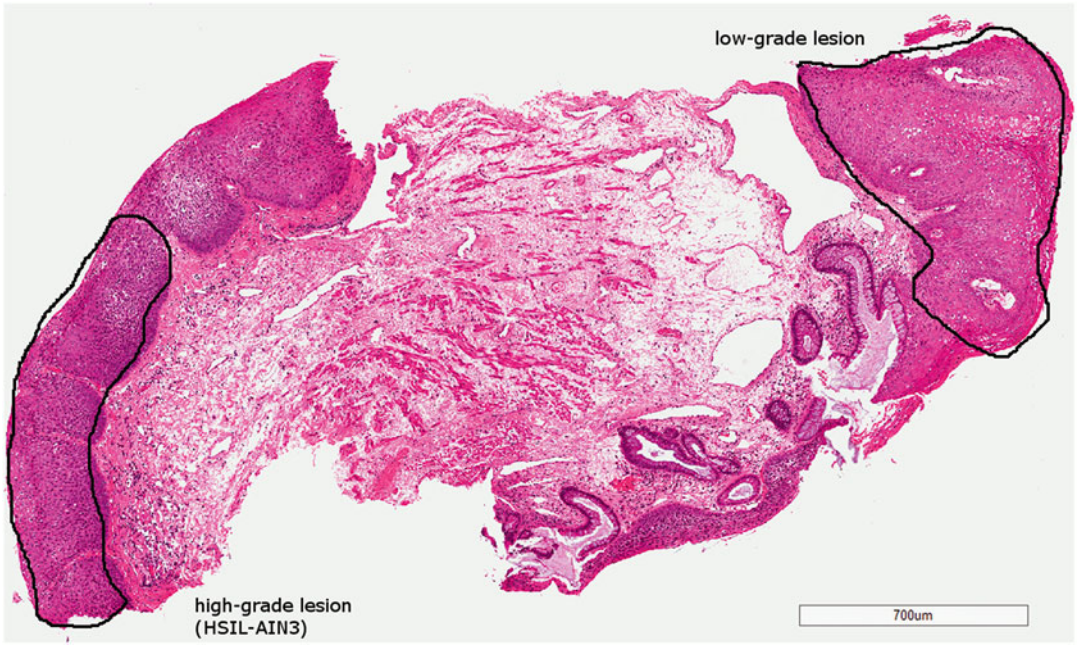


Fig. 1 Digitised image of a haematoxylin and eosin (H&E) stained biopsy section. Two lesions have been annotated using Aperio ImageScope software: a high-grade squamous intraepithelial lesion anal intraepithelial lesion grade 3 (HSIL-AIN3) and a low-grade squamous intraepithelial lesion (LSIL)

3.2 Laser Capture Microdissection

1. To dewax each PEN-membrane slide, 5 × 50 ml polypropylene tubes are required. Add 35 ml of 100% xylene to the first two tubes and 35 ml of 100% ethanol to the next two tubes. The solvent volume should be sufficient to cover the tissue region and PEN membrane, but not the slide labels.
2. Incubate the slide in each successive tube for 5 min. Use a fresh pair of disposable forceps to transfer slides between tubes.
3. After the final ethanol wash, air-dry the slides in a clean tube for 5 min. Leaving the slides in the tubes, incubate at 37 °C for an hour and leave to dry further in a container with dessicant beads overnight.
4. Refer to the annotated digitised H&E and/or p16 slides to identify areas of tissue to be captured.
5. Microdissect the area(s) of interest, capturing one lesion onto each Macro LCM cap, taking care to only capture the basal and parabasal layers—avoid capturing the superficial mucosal layer (*see* **Note 4**) (Fig. 2).
6. Cap a sterile PCR tube with each Macro LCM cap used.
7. Carefully label caps and tubes and store at 4 °C until ready to digest.

3.3 DNA Extraction

1. Perform DNA extraction in an appropriate PCR cabinet or Class II biosafety cabinet.
2. Refer to the Arcturus PicoPure DNA Extraction Kit protocol F, DNA Extraction Protocol for CapSure Macro LCM Cap Samples. Each vial of proteinase K will be sufficient

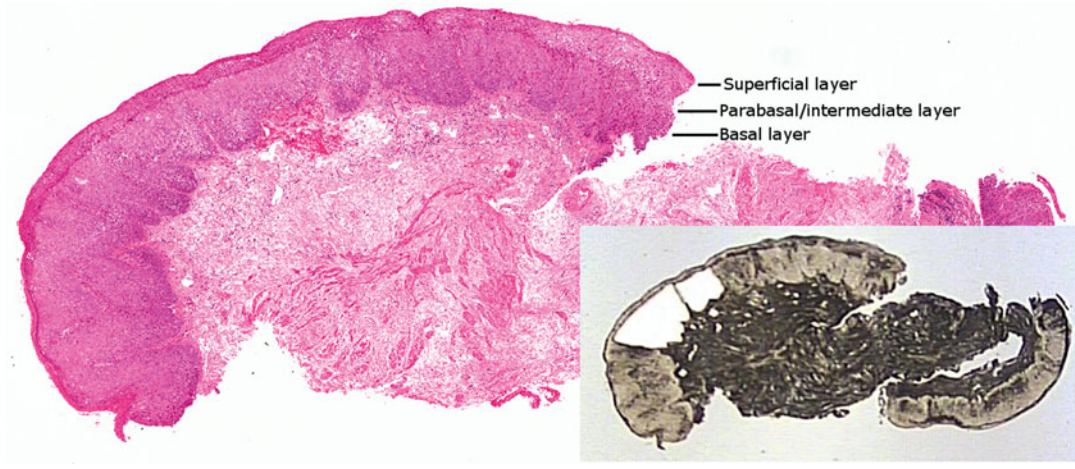


Fig. 2 Digitised image of a haematoxylin and eosin (H&E) stained biopsy section, indicating the basal, parabasal, and superficial layers of the squamous epithelium. Inset: digital photograph of an unstained section of the same biopsy following laser capture microdissection. Note that only the basal and parabasal layers were captured, leaving the superficial layer behind

for three Macro Caps. Once reconstituted, proteinase K solution cannot be stored for later use.

3. Resuspend each vial of lyophilized proteinase K with 155 μL of Reconstitution Buffer. Mix gently but thoroughly. Place the tube on ice immediately.
4. Using a microcentrifuge tube opener, carefully remove each LCM cap from its PCR tube and carefully place to the side, ensuring that the side with the captured tissue fragment is not at risk of contamination. To minimize contamination risk, remove only one LCM cap at a time.
5. If the PCR tube is cracked, discard and replace with a new sterile tube. Pipette 50 μL of reconstituted proteinase K into the PCR tube.
6. Firmly insert the LCM cap into the PCR tube and invert. Tap or shake to ensure that the cap surface is covered with proteinase K solution.
7. Incubate inverted tubes at 65 °C overnight (>16 h).
8. After incubation, remove the tubes from the incubator and centrifuge for 1 min at 1000 × *g*. Ensure that the rotor or adaptor is the correct size for the PCR tubes being used.
9. Using a microcentrifuge tube opener, carefully remove each LCM cap from its PCR tube and discard cap. Close PCR tube lid. Using a heat block, incubate at 95 °C for 10 min. If the PCR tubes do not fit snugly into heat block, first aliquot each sample into a labeled, appropriately sized microfuge tube.
10. Cool samples to room temperature. Pulse centrifuge and store at −20 °C to −80 °C when not in use.

3.4 Assessment of DNA Integrity

1. Prepare the PCR mastermix in a pre-PCR room in an appropriate PCR cabinet or Class II biosafety cabinet.
2. Aliquot quantitative beta-globin PCR mix in a 96-well LightCycler PCR plate to contain the following final components per 10 μL reaction: 1 × SensiFast Probe No-ROX mix; primers PCO3 (5′-ACACAACTGTGTTCACTAGC-3′) and PCO4 (5′-CAACTTCATCCACGTTCACC-3′) at 10 pmol each per reaction; probe PCO3_4 (5′-6-FAM-CTGCCGTTACTGCC CTGTGG-BHQ1-3′) at 2 pmol per reaction; 5 μL of DNA sample.
3. Include a no-template control and a quantitated beta-globin standard or positive control (e.g., quantitated human genomic DNA).
4. Cover the plate with transparent LightCycler adhesive seal and pulse centrifuge.

5. Perform quantitative real-time PCR on LightCycler 480 with the following cycle parameters: 95 °C for 5 min, followed by 45 cycles of (95 °C for 10 s, 60 °C for 30 s with single-data acquisition, 72 °C for 1 s) and 40 °C for 1 min. Ensure that the FAM channel/filter is selected for acquisition.
6. Analyze data on the FAM channel/filter. Samples with a positive cycle threshold (Ct) or crossing point (Cp) of 40 or lower are considered adequate.

3.5 HPV Genotyping

For mucosal/squamous cell samples, HPV genotype testing can be performed in the first instance on the RHA kit HPV SPF10-LiPA25, version 1. The majority of squamous lesions will test positive for a single genotype on this assay. Refer to the manufacturer's instructions for detailed instructions.

1. Aliquot the SPF10-LiPA25 PCR mastermix and add DNA in a pre-PCR room in an appropriate PCR cabinet or Class II biosafety cabinet.
2. Perform the amplification cycle on a standard thermocycler. Store amplicons at −20 °C.
3. Detection of amplicons can be performed manually using a water bath and a plate rocker, or on an automated blotter (Auto LiPA) and should be performed in a post-PCR room. Ensure all surfaces are decontaminated with hypochlorite bleach followed by 70% ethanol before and after each detection run.
4. If a single lesion is positive for more than one genotype, consider repeating laser capture microdissection to either side of the original captured tissue [3, 5, 6].
5. If a squamous lesion is beta-globin positive but negative on HPV SPF10-LiPA25, screen the sample PCR amplicon (there is no need to re-amplify, as both ELISA and RHA use the same PCR mastermix) with DNA ELISA kit HPV SPF10, version 1 according to the manufacturer's instructions. The ELISA is validated for over 40 HPV genotypes, and is able to detect many more.
6. If a sample is ELISA positive, repeat genotype detection of the same amplicon using SPF+ RHA strips, by the same detection method as for HPV SPF10-LiPA25. SPF+ strips contain probes for HPV26, 30, 55, 61, 62, c64, 67, 69, 71, 71sub, 82, 83, 84, 85, 87, 89, 90, and 91. If a sample is still unable to be genotyped, consider alternative methods, such as sequencing.
7. If a confirmed high-grade squamous or glandular lesion is ELISA negative, it is recommended that testing for the E6 gene be conducted for at least HPV16, 18, and 45. This is

because genomic integration may sometimes disrupt the L1 target region of the HPV genome, leading to a false negative result on L1-based assays such as SPF10 [19]. Genotype-specific E6 qPCR primer and probe sequences are as follows: HPV16 [forward primer 5′-GCACAGAGCTGCAAACAACT-3′, reverse primer 5′-AATCCCGAAAAGCAAAGTCA-3′, probe 5′-6-FAM-CGCAGTAACTGTTGCTTGCAGTA-CACA-BHQ1-3′], HPV18 [forward primer 5′-TGTGCACG GAACTGAACACT-3′, reverse primer 5′-TGCAGCATGGGG TATACTGT-3′, probe 5′-LC610-CCTCTGTAAGTTCCAA TACTGTCTTGCAA-BHQ2-3′], HPV45 [forward primer 5′-CTACAAGACGTATCTATTGCCTGTG-3′; reverse primer 5′-CCAGTGTCTCTCCATATACAGAGTTT-3′; probe 5′-Cy5-CCTCTGTGCGTTCCAATGTTGCTT-BHQ3-3′].

8. Prepare the E6 qPCR mastermix in a pre-PCR room in an appropriate PCR cabinet or Class II biosafety cabinet. Reactions can be performed as multiplex or singleplex reactions. Aliquot into a 96-well LC480 PCR plate the following 20 μL reaction mix: 1× Lightcycler 480 Probes mastermix; 1 μM of each primer; 0.2 μM each of HPV16 and HPV45 probe, 0.1 μM of HPV18 probe; 5 μL of DNA sample or genotype-specific positive control.
9. Amplify on the LightCycler 480 with the following cycle parameters: 95 °C for 10 min, followed by 55 cycles of (95 °C for 10 s, 55 °C for 30 s and 65 °C for 20 s with single-data acquisition) and 40 °C for 1 min. Apply color compensation as required.

3.6 Bisulfite Conversion for Methylation Sequence Analysis

Different methods for the conversion of unmethylated cytosine residues to uracil for methylation sequence analysis are currently used. Because LCM samples contain small quantities of fragmented DNA, it is critical to use a bisulfite conversion method that can accommodate low quantities of starting DNA, which uses reagents that protect the DNA from further degradation during the modification process, and that has very efficient bisulfite conversion to ensure consistent quality in analyzing several CpG positions during pyrosequencing. The Methylamp DNA modification kit is a fast and convenient approach for bisulfite conversion of samples containing very small quantities of DNA, including single cells, microdissection samples, paraffin-embedded tissues, plasma/serum samples, and body fluid samples.

1. DNA extracted from cervical cell lines (100 ng) and from LCM samples (1–100 ng) are to be treated using MethylAmp DNA modification Kit, as per the manufacturer's instructions with a few modifications as follows.

2. When using a new kit, add 7 ml of 100% Ethanol to the modified DNA cleaning buffer (R5) to make the final cleaning buffer (optimal for 40 reactions). Write the date on the bottle when ethanol is added.
3. Prepare 90% ethanol on the day of the conversion reaction.
4. Take into account that one vial of R2 is optimal for the treatment of ten samples. The kit comes with four vials for a total number of 40 samples.
5. Add 24 μl of DNA sample into a clean labeled tube (*see* **Note 5**). Add 1 μl of DNA denaturant buffer (R1) to the above sample. Mix well and incubate the sample at 37 °C for 10 min.
6. Add 1.1 ml of modification DNA buffer (R3) to 1 vial of DNA modification powder (R2). Vortex until the solution is totally clear (about 2 min). Add 40 μl of DNA denaturant buffer (R1) to the solution, lightly vortex. This R1/R2/R3 solution is optimal for ten DNA sample treatments (*see* **Note 6**).
7. Add 125 μl of R1/R2/R3 solution to each sample. Vortex and incubate at 65 °C for 90 min (*see* **Note 7**).
8. Place a spin column into a 2 ml collection tube. Add 300 μl of modified DNA capture buffer (R4) to each sample, mix, and transfer to the column. Centrifuge at 14,000 × *g* (12,000 rpm) for 15 s. Remove the column from the collection tube and discard the flow-through. Return the column to the collection tube.
9. Add 200 μl of final cleaning buffer (R5) to the column, and centrifuge at 14,000 × *g* (12,000 rpm) for 15 s (*see* **Note 8**).
10. Add 10 μl of R1 buffer to 1.1 ml of 90% ethanol, and mix. Add 50 μl of the mixed R1/ethanol solution to the column. Incubate for 8 min at room temperature, then centrifuge at 14,000 × *g* (12,000 rpm) for 3 min. Discard the flow-through.
11. Add 200 μl of 90% ethanol to the column, centrifuge at 14,000 × *g* (12,000 rpm) for 15 s. Remove the column from the collection tube and discard the flow-through. Return the column to the collection tube. Add 200 μl of 90% ethanol to the column again and centrifuge at 14,000 × *g* (12,000 rpm) for 3 min.
12. Place the column in a new 1.5 ml vial. Add 30 μl of R6 directly to the column filter. Incubate at room temperature for 6 min and centrifuge at 14,000 × *g* (12,000 rpm) for 3 min to elute modified DNA.
13. Modified DNA is ready for methylation amplification, or storage at −20 °C for up to 2 months (*see* **Note 9**).

3.7 DNA Purification after Bisulphite Conversion

This step is only necessary when the bisulfite conversion method used does not include a DNA purification step. Use the QIAamp DNA Mini Kit as per the manufacturer's instructions with a few modifications to increase DNA yield, including modifications to length of centrifugation and incubation in some steps, as follows:

1. Add 180 µl of lysis buffer (AL) to the sample, vortex for 15 s, and then incubate at 56 °C for 1 h.
2. Centrifuge the 1.5 ml microcentrifuge tube to remove droplets from inside the lid. It is essential that the sample and Buffer AL are mixed thoroughly to yield a homogeneous solution (*see* **Note 10**).
3. Add 200 µl ethanol (96–100%) to the sample, and vortex for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove droplets from inside the lid.
4. Carefully apply this mixture to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 × *g* (8000 rpm) for 2.5 min (*see* **Note 11**). Place the QIAamp Mini spin column in a clean 2 ml collection tube, and discard the tube containing the filtrate.
5. Carefully open the QIAamp Mini spin column and add 500 µl of washing buffer 1 (AW1) without wetting the rim. Close the cap, and centrifuge at 6000 × *g* (8000 rpm) for 2.5 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube and discard the collection tube containing the filtrate.
6. Carefully open the QIAamp Mini spin column and add 500 µl of washing buffer 2 (AW2) without wetting the rim. Close the cap and centrifuge at full speed (20,000 × *g* or 14,000 rpm) for 3 min.
7. Place the QIAamp Mini spin column in a new 2 ml collection tube and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.
8. Place the QIAamp Mini spin column in a new 1.5 ml microcentrifuge tube and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 30 µl of elution buffer (AE). Incubate at room temperature for 8 min, and then centrifuge at 6000 × *g* (8000 rpm) for 2.5 min (*see* **Note 12**).

3.8 PCR Amplification for Methylation Analysis

A strong and specific PCR product is required for a successful methylation analysis using pyrosequencing. The right amplification and sequencing primer design, analysis of bias of amplification, and high intra- and inter-assay reproducibility are crucial for reproducible results. In general, amplicons for CpG assays should ideally be shorter than 200 bp and one primer must be biotinylated at the 5′ end, to enable immobilization of the PCR product to streptavidin-

coated beads during pyrosequencing. The analysis of methylation in LCM samples has additional technical challenges due to the small amount of DNA and low sensitivity of the assays used; however, nested PCR-pyrosequencing or nested methylation-specific PCRs can increase sensitivity [16]. The procedure described here employs a sensitive and reproducible nested PCR-pyrosequencing-based method to accurately quantify methylation at ten CpG sites within the E2BS1, E2BS2,3,4 and Sp1 binding sites in the viral upstream regulatory region of the HPV16 genome in LCM samples. Four different sets of primers used in primary and nested PCR reactions, covering the E2BS1 binding site (four CpG sites at positions 7426, 7432, 7453, and 7459) and the promoter region covering the E2BS2,3,4 and Sp1 binding sites (six CpG sites at positions 7860, 31, 37, 43, 52, and 58) of the HPV16 genome have been previously reported by our group [15, 16] and are described in this protocol. Methylation sequence analysis of other regions of the viral or host genomes should follow the same principles, using nested PCR primer sets designed for the targets of interest.

3.9 Primary PCR for Amplification of the E2BS1 Region (See Note 13)

1. Thaw the HotStarTaq Master kit (Qiagen) and primer solutions in a pre-PCR room in an appropriate PCR cabinet or Class II biosafety cabinet.
2. Prepare a PCR master Mix containing the following final reagents per 25 μL reaction: 1× HotStarTaq Master mix, primers for the E2BS1 region, Ampy E2BS1 F2 Bio (5′-Bio-ATTGTGTTGTGGTTATTTATTGTA-3′) and Ampy E2BS1 R3 (5′-AACCATAATTACTAACATAAAACT-3′) at 0.25 pmol each per reaction; and DNase/RNase-free water to volume. The amplicon should be 175 base pairs long.
3. Gently pipette the master mix up and down for thorough mixing and dispense 20 μl into PCR tubes or 96-well PCR microplate.
4. Add 5 μl of bisulfite-converted DNA to the individual PCR tubes or into the 96-well PCR microplate. Include negative controls, with all of the reaction components but devoid of DNA, and methylation controls of bisulfite-modified CaSki and SiHa DNA, previously characterized as having high and low CpG methylation levels for each experiment.
5. Thermocycler conditions are as follows: denaturation for 15 min at 95 °C followed by 50 cycles of amplification consisting of 40 s at 95 °C, 30 s at 50 °C, and 40 s at 72 °C, with a final extension of 6 min at 72 °C and hold at 4 °C.
6. After amplification, the samples can be stored overnight at 2–8 °C or at −20 °C for longer storage.

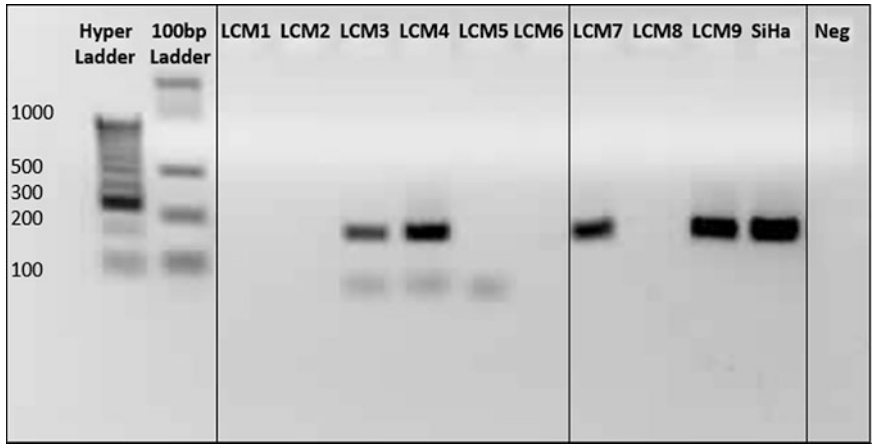


Fig. 3 Agarose gel image showing amplified products generated by primary PCR amplification of bisulfite-converted LCM-extracted DNA and SiHa cell line control DNA using the Ampy E2BS2,3,4 F/Ampy E2BS2,3,4 R bio primer pair. Expected size of amplicon is 193 bp

3.10 Primary PCR for Amplification of the E2BS2,3,4 and Sp1 Region (Fig. 3)

1. Follow the same procedure as described above, changing only the primers used to amplify the E2BS2,3,4, and Sp1 region: Ampy E2BS2,3,4 F (5′-ACATTTTATACCAAAAA ACAT-3 and Ampy E2BS2,3,4 R Bio (5′-Bio-AAATCCTAAAACATT ACAATTCTC-3′). The amplicon should be 193 base pairs long.

3.11 Nested PCR for Amplification of the E2BS1 Region (See Note 14)

1. Thaw the HotStarTaq Master kit (Qiagen) and primer solutions in a pre-PCR room in an appropriate PCR cabinet or Class II biosafety cabinet.
2. Prepare a PCR master Mix containing the following final reagents per 25 μL reaction: 1× HotStarTaq Master mix, primers for the E2BS1 region, Ampy E2BS1 F2 Bio (5′-Bio-ATTGTGTTGTGGTT ATTTATTGTA-3′) and Ampy E2BS1 R2 (5′-AACACATTTTATACCAAAAAAC-3); and DNase/RNase-free water to volume. The amplicon should be 142 base pairs long.
3. Gently pipette the master mix up and down for thorough mixing and dispense 24 μl into PCR tubes.
4. In the post-PCR room add 1 μl of each primary PCR product to the respective nested PCR Master mix. Also include 1 μl of amplified negative and positive controls. Thermocycler conditions are as follows: denaturation for 15 min at 95 °C followed by 50 cycles of amplification consisting of 40 s at 95 °C, 30 s at 56 °C, and 40 s at 72 °C, with a final extension of 6 min at 72 °C and hold a 4 °C.

3.12 Nested PCR for Amplification of E2BS2,3,4 and Sp1 Region

1. The same procedure as described above for the nested PCR must be followed changing only the primers used to amplify the E2BS2,3,4, and Sp1 region: Ampy E2BS2 F (5′-TGTATA TGGGTGTGTGTAAAT-3′) and Ampy E2BS2,3,4 R Bio (5′-Bio-AAATCCTAAAACATTACA ATTCTC-3′). The amplicon should be 184 base pairs long.

3.13 Gel Electrophoresis

1. Prepare a 2% agarose gel in 1× TAE Buffer. For a large gel, add 3 g agarose to 150 ml 1× TAE and heat to boiling in a conical flask. Allow to cool until able to hold comfortably in the hand.
2. While the gel solution is still warm, dilute GelGreen 10,000 stock reagent into the agarose gel solution at 1:10,000 and mix thoroughly.
3. Pour into a gel tray that has been prepared with combs inserted. Allow the gel to cool and set firm. Carefully remove combs.
4. Stain PCR products and DNA ladders with 6× DNA loading buffer.
5. Place gel tray in 1× TAE in an electrophoresis tank.
6. Load the stained DNA into the gel wells and apply electrodes.
7. Apply 80 V to gel for 90 min.
8. Image the stained gel with a transilluminator, a Dark Reader® or a laser-based gel scanner using a long path green filter such as a SYBR® filter or GelStar® filter.
9. Only samples that show a clear amplification product are optimal for pyrosequencing (Fig. 4).

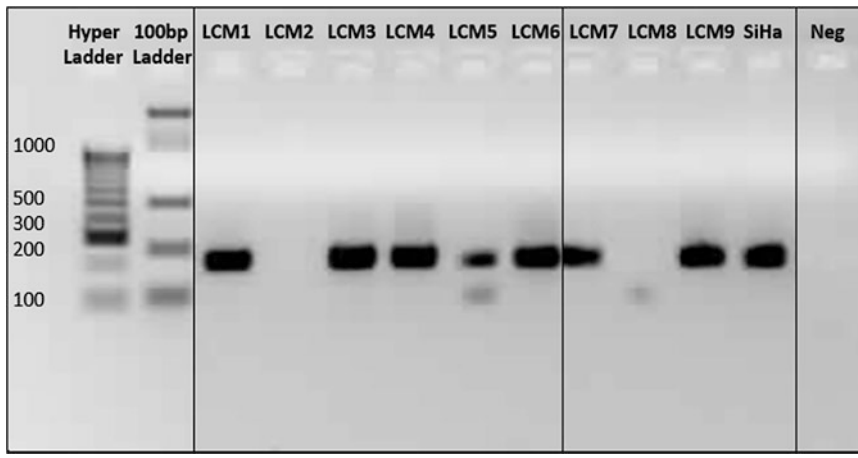


Fig. 4 Agarose gel image showing amplified products generated by nested PCR amplification of bisulfite-converted LCM-extracted DNA and SiHa cell line control DNA using the Ampy E2BS2 F/Ampy E2BS2,3,4 R bio primer pair. Expected size of amplicon is 184 bp

3.14 Methylation Analysis Using Pyrosequencing

Pyrosequencing provides a rapid, high-throughput means of detecting methylation levels at individual loci or estimating global methylation changes. This technique determines the ratio of C/T base changes after bisulfite treatment, reflecting the proportion of unmethylated and methylated cytosines at each CpG site in the original sequence [20]. To achieve this, biotinylated amplicons are immobilized on sepaharose-streptavidin beads in independent PCR plates, the DNA strands are denatured and any unlabeled DNA is removed from the sample, leaving a single labeled DNA template strand. This single-stranded template is then annealed to a shorter internal sequencing primer and the sequence is then determined by sequentially adding nucleotides to the pyrosequencing reaction [21]. For this protocol, one sequencing primer is used for the E2BS1 amplicon, and two sequencing primers are used to generate two distinct sequence products from the E2BS2,3,4 amplicon.

3.15 Immobilizing the PCR Product to Beads

1. Transfer 5–20 μL of biotinylated PCR product into each well of a new PCR plate, add 40 μL of binding buffer and 1 or 2 μL of Sepharose beads and top up to 80 μL with high purity water. Check the lot number of the Streptavidin Sepharose HP; for lot number 10057037 or higher, use 1 μl of beads, and for lot numbers lower than 10057037, use 2 μl (*see* **Note 15**).
2. Cover the plate with a sealing tape and incubate the reaction mixture for 10 min at room temperature under constant mixing (1400 rpm). It is crucial that the beads do not sediment. Ensure that no leakage is possible between the wells.
3. During immobilization, proceed with the following two steps to prepare the reaction plate and the vacuum workstation.

3.16 Separation of DNA Strands and Release of Samples into the PyroMark Q24 Plate

1. Ensure that the PyroMark Q24 Vacuum Workstation has been assembled correctly according to the manufacturer's instructions.
2. Fill five separate troughs supplied with the Workstation as follows:

   Trough 1: Approximately 50 ml ethanol (70%)

   Trough 2: Approximately 40 ml Denaturation Solution

   Trough 3: Approximately 50 ml 1× Wash Buffer

   Trough 4: Approximately 50 ml high-purity water

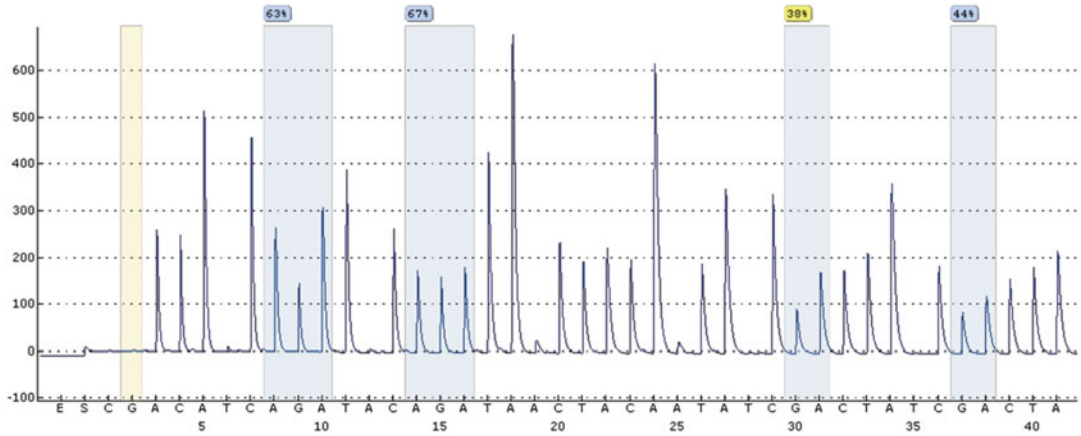
   Trough 5: Approximately 70 ml high-purity water
3. Dilute the sequencing primer to 0.375 μM in Annealing Buffer. Add 20 μl of the solution to each well of a PyroMark Q24 Plate and place it on the work table of the PyroMark Q 24 instrument.
4. Also place the PCR plate with the biotinylated PCR product immobilized to the beads on the work table. Ensure that the

plate is in the same orientation as when the samples were loaded (*see* **Note 16**).

5. The Sepharose beads, carrying the biotinylated PCR product, will be held to filter probes by vacuum and moved through the different troughs until the beads are released into the PyroMark Q24 plate as follows.
6. Apply vacuum to the tool by opening the vacuum switch.
7. Carefully lower the filter probes into the PCR plate to capture the beads carrying the biotinylated PCR product. Hold the filter probes in place for 15 s.
8. Ensure that all liquid is aspirated from the wells and that all beads are captured onto the filter probe tips (*see* **Note 17**).
9. Transfer the tool to the trough containing 70% ethanol (trough 1). Flush the filter probes for 5 s.
10. Transfer the tool to the trough containing Denaturation Solution (trough 2). Flush the filter probes for 5 s.
11. Transfer the tool to the trough containing Wash Buffer (trough 3). Flush the filter probes for 10 s. Raise the tool to beyond 90° vertical for 5 s, to drain liquid from the filter probes.
12. While holding the tool over the PyroMark Q24 Plate, close the vacuum switch on the tool (Off).
13. Release the beads in the PyroMark Q24 plate containing sequencing primer, by shaking the tool gently from side to side.
14. With the vacuum switch closed (Off), transfer the tool to the trough containing high-purity water (trough 4) and agitate the tool for 10 s.
15. Wash the filter probes by lowering the probes into high-purity water (trough 5) and applying vacuum. Flush the filter probes with 70 ml high-purity water.
16. Raise the tool to beyond 90° vertical for 5 s, to drain liquid from the filter probes. Close the vacuum switch on the tool (Off) and place the tool in the Parking (P) position.

3.17 Annealing of Sequencing Primer to Samples and Pyrosequencing

1. Incubate the PyroMark Q24 Plate containing the beads carrying the biotinylated PCR product and the sequencing primer at 80 °C, for 5 min, using a PyroMark Q24 Plate Holder.
2. During the incubation prepare the PyroMark Q24 Cartridge with the required nucleotides, enzyme mixture, and substrate (*see* **Note 18**).
3. Place the PyroMark Q24 Cartridge and the PyroMark Q24 plate into the PyroMark instrument and start the run (*see* **Note 19**).



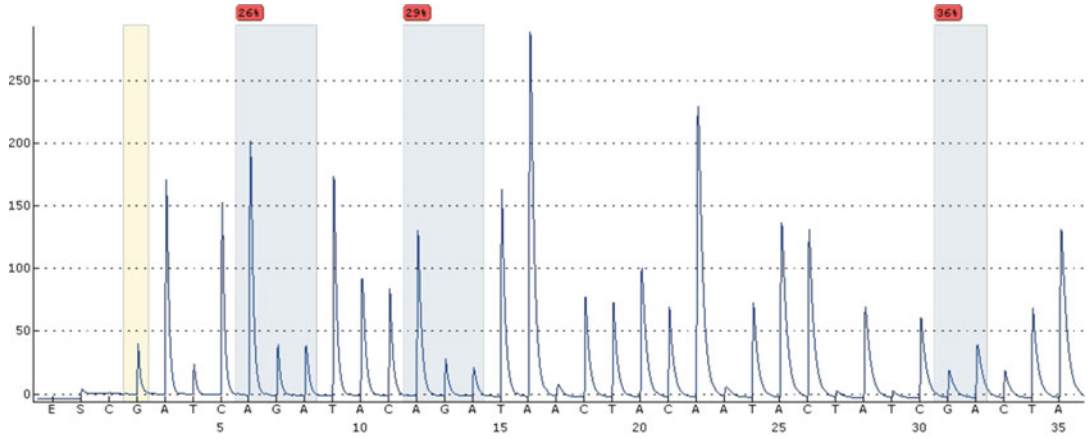
Sequence to analyze:
ACAACCRAATTCRATTAAAACTACAAAATAACCRCTAACRCTACAAAATATAATATATATAAAAC

Position	1	2	3	4
Quality	Passed	Passed	Check	Passed
Meth (%)	63	67	38	44

Warnings:
Position 3:
-Uncertain due to high sum deviation in variable position.

Fig. 5 Representative pyrogram showing methylation levels at four CpG sites in a LCM sample using nested amplified products. CpG sites are highlighted with a blue-gray background colour, and bisulphite treatment controls in CpG assays with a light orange background colour. The methylation percentage is displayed above the corresponding CpG site. Percentages in blue indicate "Passed quality" while yellow indicates "Check quality." Warnings and errors are indicated at the bottom of the pane

4. Analyze the results using the PyroMark 24 Advanced Software. The methylation percentage is shown in the Pyrogram pane above the corresponding specific CpG site analyzed and the quality assessment is indicated by the background color of the result. The quality color is an indicator of the overall quality of the assay, as well as the reliability of the data. A blue color indicates "passed quality" showing that the assay performed well and all controls are within required limits (Fig. 5). A yellow color indicates "check quality" and shows that one or more inconsistencies were detected during the run (Fig. 5). Detailed information of the problems can be found in the well window. The data could be acceptable if the reason for the inconsistency can be determined and if the data are not compromised. A red color indicates failed quality (Fig. 6). This could indicate that the overall signal is too low, that peak heights vary broadly from the expected value, that peak patterns are different to the sequence to analyse, or that a strong signal was detected in a blank control dispensation. Detailed information of the problems can be found in the well window. A white color indicates



Sequence to analyze:
AACCRAATTCRATTAAAACTACAAAATAAACCTAACRCTACAAAATATAATATATATAAAAC

Position	1	2	3
Quality	Failed	Failed	Failed
Meth (%)	26	29	36

General warnings:
-Failed bisulfite conversion at dispensation: 2.

Warnings:
Position 1:
-Failed surrounding reference sequence pattern.
-Uncertain due to high pattern deviation in variable position.
Position 2:
-Failed surrounding reference sequence pattern.
Position 3:
-Failed surrounding reference sequence pattern.

Fig. 6 Representative pyrogram showing methylation levels at four CpG sites in a LCM sample using nested amplified products. CpG sites are highlighted with a blue-gray background colour, and bisulphite treatment controls in CpG assays with a light orange background colour. The methylation percentage is displayed above the corresponding CpG site. Percentages in red indicate "Failed quality." Warnings and errors are indicated at the bottom of the pane

"not analyzed" if the analysis is not supported by the software or the variable position has been deselected. Bisulphite treatment controls in CpG assays are highlighted with a light orange background color.

4 Notes

1. Acid alcohol solution has a 3 month expiry, remake solution if not used by this time. Hydrochloric acid is corrosive on ingestion, to the eyes and skin. It is an irritant on inhalation. It should be stored in a corrosives cupboard. Small volumes may be disposed of down the sink with copious quantities of water. When mixing concentrated acids and water, always add the acid to the water to avoid the dangers of a vigorous exothermic reaction.

2. Xylene is flammable and harmful by inhalation and by contact to the skin. Only use polypropylene tubes and rack holders and take care when decanting xylene. Place absorbent paper towelling over work area. Clean up any small spills immediately with water and paper towels. Refer to institutional procedures for large spills. Use xylene only in an appropriate chemical fume hood and dispose all xylene waste as per institutional protocol.
3. For brevity the complete tissue staining protocols have not been included, as these are standard stains routinely performed in most histopathological laboratories.
4. It is highly recommended that a visual record of each microdissection is recorded, i.e., acquire static images of each section before and after microdissection, as well as the tissue fragment on the cap. Carcinomas may not have an obvious basal/parabasal/superficial layer structure; in this case, simply avoid capturing the outer edge of the biopsy section. Use a single Macro LCM cap for each lesion.
5. If the DNA sample is very concentred (>100 ng), use 14 μl of DNA plus 10 μl of distilled H_2O.
6. When mixing reagents R3 and R2, be sure that the solution is completely clear. The prepared R1/R2/R3 solution should be used immediately; do not leave or store any remnant of this solution for later DNA modification.
7. The DNA will be poorly modified if the sample is not incubated at the correct temperature.
8. Ensure that 100% ethanol was added into R5 before using each new kit. If <100% ethanol is added, the elution will contain little or no DNA.
9. We have observed that modified DNA can be stored for up to 9 months at −20 °C without a decrease in methylation sequence quality.
10. A white precipitate may form on addition of Buffer AL, but it will not interfere with the QIAamp procedure.
11. Centrifugation at full speed will not affect the yield or purity of the DNA. If the solution has not completely passed through the membrane, centrifuge again at a higher speed until all the solution has passed through.
12. For long-term storage of DNA, eluting in Buffer AE and placing at −30 °C to −15 °C is recommended.
13. Successful amplification of both target regions will be observed for approximately 20% of the LCM samples using only primary PCR amplification.

14. Successful amplification of all target regions will be observed for approximately 85% of the LCM samples using nested PCR amplification.
15. In general, 5 μL of PCR product is enough but depending on the strength of the PCR product band observed on the agarose gel, more or less product could be added. Too little PCR product will lead to insufficient peak height for accurate quantification in the pyrogram, while too much PCR product might lead to loss of signal due to premature depletion of pyrosequencing reagents.
16. Sepharose beads sediment quickly. Capturing of beads must take place immediately following agitation. If more than 1 min has elapsed since the plate was agitated, agitate again for 1 min before capturing the beads.
17. If the wells still contain liquid or white beads remain, the filter probes may need replacing.
18. Reagents must be at ambient temperature (20–25 °C). The amounts of reagent needed for a run are indicated by the PyroMark Q24 Advanced software. It is recommended that the reagent cartridge be used a maximum of 30 times.
19. The time between removing the plate holder from the heating block and placing the PyroMark Q24 plate in the PyroMark Q24 Instrument and starting the run should not exceed 30 s.

Acknowledgments

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Chapter 10

Laser Capture Microdissection and Transcriptional Analysis of Sub-Populations of the Osteoblast Lineage from Undecalcified Bone

Efrain Pacheco, Rong Hu, and Scott Taylor

Abstract

Transcriptional analysis of tissue samples is a useful and widely applied approach to provide insight into the molecular mechanisms engaged in response to phenotypic changes or external stimuli. We describe a method that overcomes the technical challenges associated with the application of Laser Capture Microdissection to undecalcified bone enabling us to collect high-quality bone tissue with maintained cellular morphology that is suitable for cryosectioning, fixation, and cutting. Using this method, we obtain samples enriched for specific cell types from the mature osteoblast lineage (osteoblasts, lining cells, i.e., quiescent osteoblasts, and osteocytes). RNA is well preserved and following extraction and amplification can be used as input to both low and high-throughput RNA analysis formats.

Key words Bone, Laser capture microdissection (LCM), Osteoblast, Osteocyte, Microarray, qRT-PCR, RNA amplification, RNA extraction

1 Introduction

Bone and cartilage are structurally complex tissues comprised of heterogeneous cell populations that regulate the growth and maintenance of the skeleton. Recent data also support the role of bone as an endocrine organ and a regulator of hematopoiesis [1–3]. There is significant cross-talk between bone cell populations with the osteocyte now being recognized as a major regulator to effect these diverse functions [4, 5]. Molecular analyses to gain insight into the role of various bone cell populations in these processes are confounded by the cellular heterogeneity of bone and associated bone marrow. Laser Capture Microdissection (LCM) is a powerful tool that can be exploited to enrich for specific bone cell populations for molecular analyses [6]. However, it has had limited application for transcriptional profiling of bone due to several technical challenges. These include preparation of high-quality undecalcified cryosections that preserve RNA quality and robust RNA

Graeme I. Murray (ed.), *Laser Capture Microdissection: Methods and Protocols*, Methods in Molecular Biology, vol. 1723, https://doi.org/10.1007/978-1-4939-7558-7_10,

amplification methods due to the limited quantity of RNA typically obtained from specific bone cell populations.

We have recently employed LCM to enrich for subpopulations of the mature osteoblast lineage (osteoblasts, lining cells, and osteocytes) from rat vertebral cancellous bone to investigate the molecular response of these cells to an anti-sclerostin antibody, a potent stimulator of bone formation [7, 8]. We used in vivo fluorochrome labeling to locate and facilitate capture of osteoblasts and lining cells. All the steps in this procedure were optimized to obtain high-quality RNA and ensure high fidelity during RNA amplification. This chapter provides the detailed materials and protocols that will allow investigators to perform laser capture microdissection on bone or other mineralized tissues for RNA analyses. We also discuss practical usage of quality control metrics relevant to the microarray data.

2 Materials

2.1 Tissue Collection

1. Dissection tools.
2. Tissue-Tek O.C.T (Optimal Cutting Temperature Medium).
3. Peel-A-Way freezing molds.

2.2 Cryosectioning

1. Slide adhesive (Leica CryoJane® Tape Transfer System).
2. Glass slides (Leica CryoJane® Tape Transfer System).
3. Adhesive Tape Windows (Leica CryoJane® Tape Transfer System).
4. Cryostat tissue disks.
5. Tungsten-carbide knives.
6. Arcturus® PEN slides (Applied Biosystems).
7. Charged slides.
8. CryoJane® Protective oil (Leica Microsystems).
9. CryoJane® 3P Solution for B for coating (Leica Microsystems).

2.3 Fixation and Dehydration

1. LCM Staining Kit (Ambion™).
2. Nuclease-Free Water.
3. Molecular biology grade ethanol, 100%.
4. Xylene.
5. Pap-Jars dehydration chambers.

2.4 LCM

1. Arcturus XT™.
2. CapSure™ LCM Macro caps.
3. Microcentrifuge tubes.

2.5 RNA Extraction

1. Arcturus® PicoPure® RNA Isolation Kit (Thermo Fisher Scientific).
2. RNase-Free DNase Set (QIAGEN Inc).
3. RNase-free Microfuge Tubes, 1.5 ml.
4. Microcentrifuge.

2.6 RNA Quality Control

1. RNA 6000 Pico Kit (Agilent Technologies).
2. 2100 Bioanalyzer (Agilent Technologies).
3. 2100 Expert Version B.02.08.SI648 Software (Agilent Technologies).
4. RNase-free Water.
5. Quant-iT™ RiboGreen® RNA Assay Kit (Thermo Fisher Scientific).
6. Amber Microfuge Tubes, 1.5 ml.
7. NanoDrop™ 3300 Fluorospectrometer (NanoDrop).

2.7 RNA Pre-amplification and TaqMan®

1. CellsDirect™ One-Step qRT-PCR Kit (Thermo Fisher Scientific).
2. SuperScript® III Reverse Transcriptase (Thermo Fisher Scientific).
3. Custom PreAmp Primer/Probe Mix (0.2×) (Thermo Fisher Scientific).
4. RNase-free Microfuge Tubes, 1.5 ml.
5. RT-PCR Grade Water.
6. PCR Plates.
7. PCR Thermocycler.
8. Custom TaqMan® Array Micro Fluidic Cards (Thermo Fisher Scientific).
9. TaqMan® Universal PCR Master Mix (Thermo Fisher Scientific).
10. Centrifuge (Sorvall Legend RT, Thermo Fisher Scientific).
11. 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific).

2.8 RNA Amplification and Microarray

1. Arcturus® RiboAmp® HS PLUS Kit (Thermo Fisher Scientific).
2. Turbo Labeling™ Kit – Biotin (Thermo Fisher Scientific).
3. RNase-free Microfuge Tubes, 1.5 ml.
4. PCR plate.
5. PCR Thermocycler.
6. Microcentrifuge.

7. Centrifuge.
8. NanoDrop 1000 UV-Vis Spectrophotometer (NanoDrop).
9. Affymetrix GeneChip® Rat Genome 230 2.0 Array (Affymetrix).
10. GeneChip® Hybridization Oven 640 (Affymetrix).
11. GeneChip® Fluidics Station 450 (Affymetrix).
12. EukGE-WS2v5_450 script (Affymetrix).
13. GCS 3000 7G with AutoLoader (Affymetrix).

2.9 Microarray Data Analysis

1. R statistical computing platform (version 3.0.1 or higher).
2. Bioconductor (version 2.14 or higher).

3 Methods

3.1 Tissue Collection

1. RNA degradation starts immediately after animal death. Minimizing the time between euthanasia and tissue freezing is essential for maintaining high-quality RNA. At necropsy, removal of tissues for downstream genomic analysis should be prioritized for prompt (<15 min) freezing.
2. Carefully clean the bones of surrounding soft tissues. Samples may be carefully trimmed with bone cutting forceps to facilitate embedding and cryosectioning.
3. Place the sample in an appropriate sized freezing mold such as Peel-A-Way molds and completely immerse in room temperature O.C.T.
4. Using long forceps, carefully immerse the freezing tissue mold containing the bone in liquid nitrogen. Gently agitate the mold side-to-side to break the vapor barrier formed around the freezing mold. O.C.T. is clear at room temperature and turns white as it freezes. Immediately after all the O.C.T. has turned white and solidified, remove from liquid nitrogen and cover with dry ice for transport to a freezer. Care must be taken not to leave the O.C.T. block in liquid nitrogen for additional time after it has completely solidified. Exposing block to room temperature while transferring it to dry ice will result in cracking in the block. If this occurs, the block can be repaired using CryoJane Protective Oil which avoids freeze-thaw artifact. We have determined liquid nitrogen is superior for snap-freezing bone compared with other freezing methods (e.g., dry ice chilled isopentane) yielding excellent preservation and cellular morphology.
5. Frozen O.C.T. blocks should be stored at −80 °C until ready for cryosectioning.

3.2 Cryosectioning

1. The use of adhesive coated slides and a Leica CryoJane system is essential to obtaining undecalcified bone sections appropriate for LCM.
2. Although ready-to-use adhesive coated slides are commercially available, we have found that freshly prepared slides are far superior for the preparation of undecalcified bone sections. (*See* **Notes 1–4** on CryoJane System and adhesive application to standard and PEN LCM slides).
3. Adhesive coated slides should be acclimated to the cryostat chamber temperature for adequate tissue transfer.
4. The ideal cutting temperature for bones is −22 to −26 °C. Transfer and acclimate the O.C.T. blocks to the temperature in the cryostat before removing the block from the mold.
5. Carefully remove the O.C.T. tissue block from the mold and mount the O.C.T. on an appropriate size cryostat tissue disk by applying a small amount of room temperature O.C.T. to bind the back of the O.C.T. block to the disk.
6. The use of a very sharp non-disposable tungsten carbide knifes is essential for sectioning undecalcified bones (*see* **Notes 1–4**).
7. Small and gradual advancing of the O.C.T. block to expose the appropriate tissue plane (tissue facing) will prevent damage to the block. We have found that a setting of 10 μm or less is appropriate for facing into undecalcified bone.
8. 4–7 μm cryosections of bone are appropriate for effective LCM. We have found the use of IR laser (contact) to be inefficient at bone thickness of 8 μm or more and 5 μm or more for UV laser (cutting).
9. Position the CryoJane adhesive tape window to the surface of the O.C.T. block. Slowly cut a tissue section holding an edge of the window with forceps.
10. Transfer the CryoJane adhesive tape window (containing the bone slice) to the adhesive coated slide and use the roller supplied with the system to flatten the section on the slide.
11. Expose the mounted tissue on the adhesive slide to one or two UV flashes to activate cross-linking of the adhesive.
12. Carefully remove the adhesive tape window by pulling it back leaving the transferred tissue sample on the slide. If complete transfer was successful, the CryoJane adhesive tape window should not contain any tissue.
13. Keep the slide in the cryostat until ready for fixation.
14. We have found that if the sections prepared using the CryoJane system are stored for longer than 12 h, there is incomplete lifting of targeted tissue by contact LCM.
15. After sectioning is complete, the O.C.T. block face is coated with Protective Oil which avoid freeze-thaw artifact.

3.3 Fixation and Dehydration

1. Prepare all alcohol grades using molecular grade ethanol (ETOH) and RNase-free water.
2. Prefill all the dehydration chambers (e.g., plastic Pap-Jars, use only once) with the different grades of ETOH and xylene, label and organize in a sequential order as follows:

   75% ETOH; 30 s

   50% ETOH; 30 s

   Cresyl Violet stain or Acridine Orange Stain (optional, applied with a disposable pipette); 5–10 s

   50% ETOH; 30 s

   75% ETOH; 30 s

   95% ETOH; 30 s

   100% ETOH; 30 s

   100% ETOH; 30 s

   Xylene; 5 min

   Xylene; 5 min

   Xylene (optional); up to 4 h
3. We have observed minimal RNA degradation for up to 4 h once the tissue is completely dehydrated and left immersed in the third xylene station.

3.4 LCM

1. Remove the dehydrated section from the last xylene station and air-dry for 5 min.
2. Active bone forming surfaces can be identified by using in vivo fluorochrome labeling which is incorporated at the site of active bone formation and mineralization as described (*see* Fig. 1. and **Notes 1–4**). Bone surfaces lacking fluorochrome labeling are identified as non-forming surfaces which would be largely covered by lining cells. Osteocytes are collected from the cortical bone surface of the diaphysis (long bones) or the trabecular bone (vertebrae).
3. Captured samples are collected on CapSure LCM macro caps and immediately transferred to a 0.5 ml micro-centrifuge tube containing 50 μm of lysis buffer (*see* **Note 5**).
4. We have determined that the immersed sample can be stored at room temperature for up to 4 h.
5. Incubate the sample at 42 °C for 30 min.
6. Samples should be stored frozen at −80 °C until ready for RNA extraction.

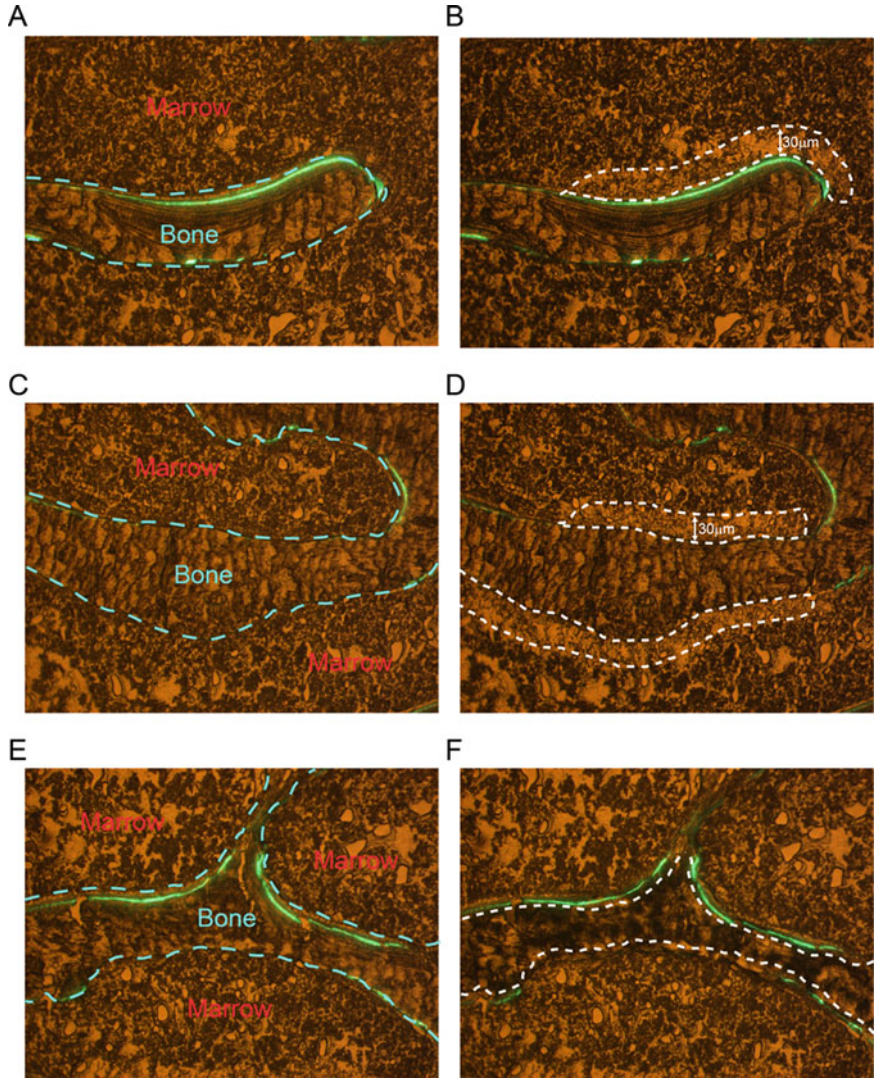

Fig. 1 (**a**–**f**) Photomicrographs illustrating laser capture microdissection of three subpopulations of the mature osteoblast lineage. (**a**) Vertebral trabecula with incorporated calcein label identifying an active forming bone surface. (**b**) Same field following microdissection capturing the tissue directly interfaced with the labeled surface, within approximately 30 mm of the bone surface, to enrich for osteoblasts (dotted white line). (**c**) Trabecula with most surfaces lacking an incorporated calcein label considered inactive bone surfaces. (**d**) Same field following microdissection capturing the tissue directly interfaced with inactive bone surfaces to enrich for lining cells (dotted white line). (**e**) Trabecula with both active and inactive surfaces for capture of osteocytes from the bone matrix. (**f**) Same trabecula viewed through the capture cap after laser application over the bone matrix (white dotted line indicates area of laser application). The dark mottling over the bone matrix indicates contact of the cap film to the bone. Although the bone matrix itself does not lift from the slide during laser capture, the successful recovery of osteocytes was confirmed via enrichment of osteocyte-specific genes. Original magnification 560× for all the samples. Figure reused from Nioi et al. [6] under the Creative Commons license

3.5 Total RNA Extraction

1. Total RNA from LCM caps is isolated using Arcturus® PicoPure® RNA Isolation Kit following the manufacturer's instructions, with the specific adaptations listed below.
2. On-column DNase treatment is performed after first column wash with Wash Buffer 1 (W1). 10 μl DNase I Stock Solution is mixed with 30 μL Buffer RDD (provided with Qiagen's RNase-Free DNase Set) by gently inverting. All the subsequent steps are performed following the kit's user guide.
3. An extra centrifugation of column at 16,000 × *g* for 1 min after the second wash with Wash Buffer 2 (W2) is carried out to further remove residual wash buffer.
4. The final elution volume is 11 μl.

3.6 Total RNA QC

1. Total RNA quality and concentration are examined using Agilent RNA 6000 Pico Kit and Quant-iT™ RiboGreen® RNA Assay Kit following the manufacturer's instructions.
2. 1 μl of total RNA is used to run Agilent RNA 6000 pico chip for each LCM cap sample for RNA quality examination following the manufacturer's instructions (Fig. 2). RNA from multiple [2–5] LCM caps of the same bone section with RNA Integrity Number (RIN) >5 are pooled.
3. 1.5 μl of pooled total RNA is used in RiboGreen RNA Assay to measure concentration.

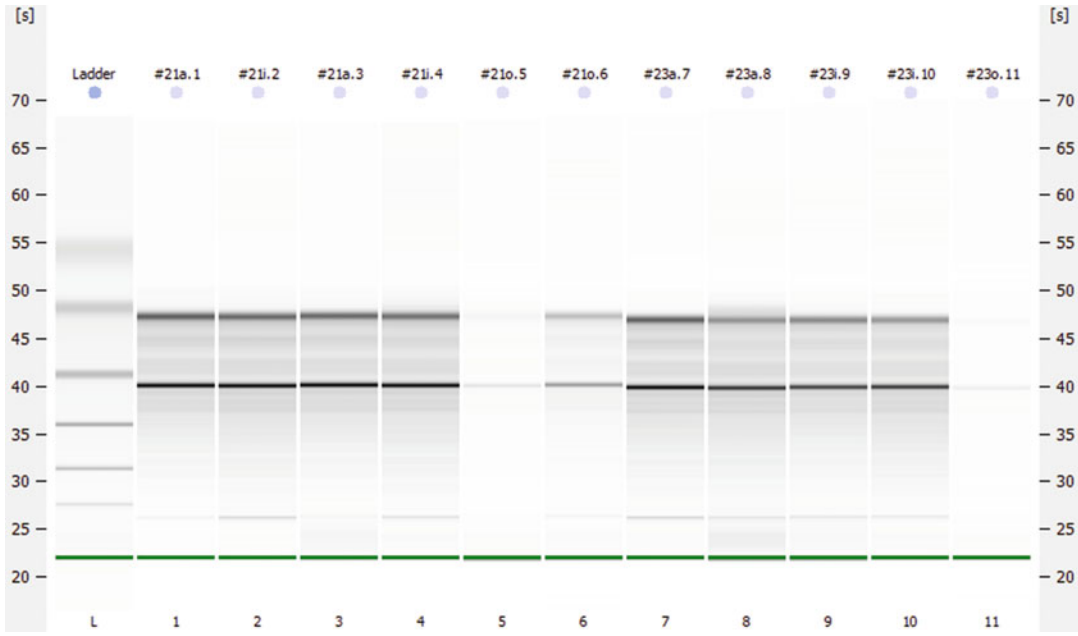

Fig. 2 Quality assessment of RNA extracted from LCM samples. Densitometric visualization illustrates intact 18S and 28S ribosomal RNA bands. Figure reused from Nioi et al. [6] under the Creative Commons license

3.7 Total RNA Amplification for TaqMan®

1. Mix 1 ng total RNA with 5 μl CellsDirect 2× Reaction Mix, 2.5 μl 0.2× Primer/Probe Mix, 0.2 μl SuperScript™ III RT/Platinum® Taq Mix and RNase-free water to a final 10 μl reaction volume per well in a PCR plate.
2. Incubate the PCR plate in a thermal cycler with program: 1 cycle of 50 °C for 15 min, 1 cycle of 95 °C for 2 min, 18 cycles of 95 °C for 15 s and 60 °C for 4 min.
3. Proceed immediately to TaqMan reaction setup, or store at −20 °C until use.

3.8 qRT-PCR with TaqMan® Array Micro Fluidic Cards

1. Mix 2 μl of the amplification product from Subheading 3.7, **step 3** with 48 μl of RT-PCR grade water and 50 μl TaqMan® Universal PCR Master Mix.
2. Load the 100 μl mixture into each fill reservoir of a customized TaqMan® Array Micro Fluidic Card and run on 7900HT Fast Real-Time PCR system according to the manufacturer's user guides.

3.9 TaqMan Data Analysis

1. The geometric mean of housekeeping gene expression levels is used for normalization, and the relative gene expression level is calculated using the $2^{-\Delta\Delta CT}$ method [9].
2. Plot TaqMan results for genes with known expression in osteoblast lineage cell types to observe differences in gene expression between the different enriched cell populations (Fig. 3).

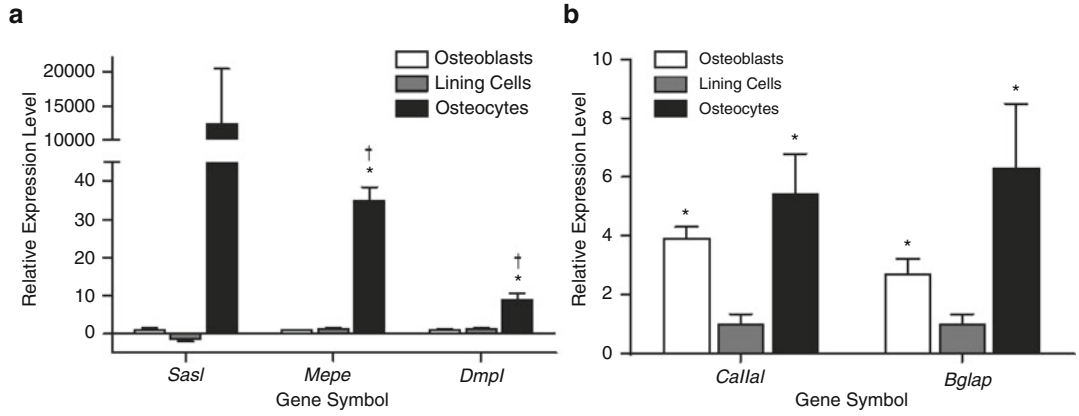

Fig. 3 (**a**, **b**) Genes highly expressed in cell types from the Osteoblast lineage are quantified using TaqMan to confirm the successful capture of tissue samples enriched for specific cell types (**a**) Osteocyte-specific genes (Sost, Mepe, and Dmp1) and (**b**) bone matrix genes Col1a1 and Bglap. Data mean + SEM are normalized to the lining cell group mean. *$p < 0.05$ vs. lining cells, †$p < 0.05$ vs. osteoblasts. Figure reused from Nioi et al. [6] under the Creative Common license

3.10 Total RNA Amplification for Microarray

1. Total RNA amplification for microarray is performed using Arcturus® RiboAmp® HS PLUS RNA Amplification Kit following the manufacturer's instructions with 1.5 ng total RNA input.
2. Concentration of amplified antisense RNA (aRNA) is determined by using 1.5 μl of the amplification product on NanoDrop 1000 following the user manual.
3. Proceed immediately to Biotin-labeling, or store the aRNA at −80 °C until use.

3.11 aRNA Labeling with Biotin

1. 15 μg of aRNA is used in the Biotin labeling reaction following the user guide of Arcturus Turbo Labeling™ Kit – Biotin. The labeled aRNA is sent to a contract lab for further microarray analysis.

3.12 Microarray Hybridizations

1. Biotin-labeled aRNA products are fragmented and then hybridized to the Affymetrix GeneChip Rat Genome 230 2.0 Array (Affymetrix, Santa Clara, CA, USA) overnight at 45 °C for 16 h in a GeneChip Hybridization Oven 640.
2. Array chips are subsequently stained, washed, and scanned using a GeneChip Fluidics Station 450, EukGE-WS2v5_450 script, and GCS 3000 7G with the AutoLoader (Affymetrix).
3. The scan files are processed using Expression Console (Affymetrix) for quality control, and subsequent results are used in microarray data analysis.

3.13 Microarray Data Analysis

1. Review standard Affymetrix RNA (Actin 3′/5′ ratio, GAPDH 3′/5′ ratio), hybridization (background intensity, percent present), and signal distribution (scale factor) quality metrics.
2. Exclude quality outliers from further analysis (*see* **Note 6**).
3. Normalize using an accepted method such as Robust Multi-Array Average implemented in the Bioconductor package *affy*.
4. Perform PCA analysis using implementations from the R *stats* package or a specialized R package such as *FactoMineR*.
5. Plot the top principal components and observe distinct clustering of samples enriched for each cell population (Fig. 4).
6. Proceed with additional data analysis.

4 Notes

1. Visualization of active bone forming surfaces can be facilitated by administration of fluorochrome labels at specific time points prior to necropsy. Calcein green, xylenol orange, and alizarin complex one are examples of fluorochrome available that are

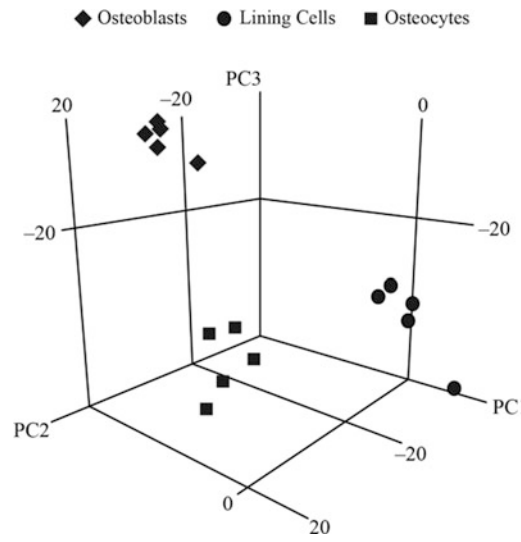

Fig. 4 Plot of top three components identified through principal components analysis of genome-wide transcription data further highlights the successful separation of enriched cell populations of the osteoblast lineage achieved using LCM techniques. Figure reused from Nioi et al. [6] under the Creative Commons license

incorporated at sites of active bone formation and mineralization (i.e., active osteoblasts). The use of these fluorochromes facilitates LCM by not requiring additional detections methods (e.g., immunohistochemistry) and sample handling prior to LCM [6].

2. Extremely sharp microtome knives are essential for the preparation of high-quality undecalcified bone cryosections for LCM. We use non-disposable D profile tungsten carbide knives. We have found that the most effective method to ensure the knife is sufficiently sharp is to sharpen these knives using Shandon Autosharp Microtome Knife sharpener. Commercial sharpening services can be used as an alternative but we have frequently found that more frequent sharpening of the knives was necessary to ensure appropriate sample quality for LCM.
3. Follow Leica's recommendation for application of CryoJane System Adhesive solution. We have found that the use of solution A was not necessary as long as clean slides were used fresh out of the packaging.
4. LCM PEN slides can be used to collect large areas of samples (i.e., cortical bone for osteocyte enriched sample). We followed the same recommended instructions to apply CryoJane System Adhesive solution to LCM PEN slides.

5. Following transfer to the micro-centrifuge, we have determined that the immersed sample can be stored at room temperature for up to 4 h.
6. Focus on consistency of QC metrics within the data set and do not rely only on the manufacturer's recommended thresholds. Observed values are likely to fall outside of suggested ranges. One simple approach is to calculate the mean and standard deviation and exclude samples with metrics that are more than 2 or 3 standard deviations from the mean.

Acknowledgments

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Chapter 11

Cell Type-Specific Laser Capture Microdissection for Gene Expression Profiling in the Human Brain

Sarah A. Mauney, Tsung-Ung W. Woo, and Kai C. Sonntag

Abstract

Cell type-specific laser microdissection technologies in combination with molecular techniques to determine gene expression profiles have become powerful tools to gain insight into the neurobiological basis of neural circuit disturbances in various neurologic or psychiatric diseases. To identify specific cell populations in human postmortem brain tissue, one can use the inherent properties of the cells, such as pigmentation and morphology or their structural composition through immunohistochemistry (IHC). Here, we describe the isolation of homogeneous neurons and oligodendrocytes and the extraction of high-quality RNA from these cells in human postmortem brain using a combination of rapid IHC, Nissl staining, or simple morphology with Laser Capture Microdissection (LCM), or Laser Microdissection (LMD).

Key words Laser capture microdissection, Laser microdissection, Postmortem brain, Immunohistochemistry, Neurons, Oligodendrocytes, Expression profiling

1 Introduction

Gene or noncoding (nc)RNA expression profiling of neural cell populations from postmortem brains has been a quickly expanding field in neuroscience and recent developments have introduced laser-assisted microdissection to individually isolate these cells. This cell-specific-based analysis enables molecular fingerprinting, such as mRNA or noncoding (nc)RNA expression profiling, without the confounding effects of surrounding cells and/or tissue structures [1, 2].

A requirement for laser microdissection on postmortem brain material usually is the availability of so-called high-quality tissue samples as determined by RNA integrity numbers (RIN) that have been generated with an Agilent bioanalyzer [2, 3]; however, our research has shown that reliable data can also be obtained from tissue with lower RINs. Another requirement is the reliable visualization of the desired neural cell populations. Some of the methods that are commonly used to identify specific subsets of neural cells

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include immunohistochemistry (IHC) and Nissl staining. However, these procedures are conventionally optimized for the purpose of preservation of morphological details for downstream neuroanatomical analyses and typically involve a number of aqueous processing steps during a rather prolonged time interval (up to 2–3 days). As a result, they can lead to significant degradation of RNA and hence are less suitable for being directly adapted for laser microdissection.

Expression profiling from laser-isolated cells requires the acquisition of high-quality mRNA or other ncRNA molecules, e.g., for a sample purity as expressed by an absorption ratio (A_{260}/A_{280}) between 1.8 and 2.1. While for microarray hybridization usually quantities in the microgram range are needed, usually obtained after two rounds of T7-based linear amplification [4], whole genome RNA-Seq can be performed from a small amount of material in the picogram range, e.g., in single-cell analysis [5]. Here, we describe three methods to identify neurons and oligodendrocytes of interest from postmortem human brains (Fig. 1): (1) A quick Nissl staining protocol to detect pyramidal neurons by their characteristic morphology [6], (2) a rapid IHC protocol to visualize parvalbumin (PV)-containing GABAergic neurons and oligodendrocytes [7, 8] and (3) the capture of naturally pigmented neuromelanin-containing dopamine neurons [9, 10]. We then describe two currently available laser-assisted methods to isolate cells in postmortem human brains—laser capture microdissection (LCM), which makes use of a low-intensity infrared laser beam to attach neurons on a matrix [11], and laser microdissection (LMD) that removes cells with a high-intensity UV cutting laser and collects them by gravity [12]. Finally, we provide information from Agilent bioanalyzer analysis about the quality of RNA isolated from captured cells for downstream applications such as quantitative real-time (qRT)-PCR, microarray hybridization, or RNA-Seq.

2 Materials

2.1 Tissue Preparation

1. RNase Zap® (Ambion®, TX).
2. Microm HM 505E cryostat (Thermo Scientific, MA).
3. Plain, uncharged glass slides for LCM (MDS Analytical Technologies, CA), or Leica Frame slides PET-membrane 1.4 μm for LMD.

2.2 Morphological Identification of Pyramidal Neurons with a Nissl Stain Procedure

1. HistoGene® LCM Frozen Section Nissl Staining Kit (MDS Analytical Technologies, CA). This kit contains not only the Nissl stain, but also all the appropriate ethanols, xylene, and slide jars for the process. The slide jars can be reused after every

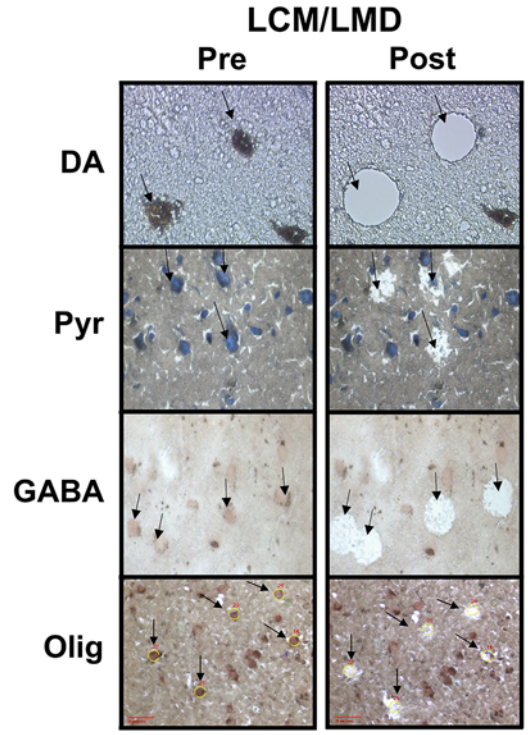

Fig. 1 Examples of neurons or oligodendrocytes before (Pre, left-hand side) and after (Post, right-hand side) laser microdissection (DA) or laser capture microdissection (Pyr, GABA, Olig) from human postmortem brain tissue. DA = dopaminergic neurons, Pyr = pyramidal neurons, GABA = Parvalbumin-expressing, γ-aminobutyric acid neuron, Olig = 2′,3′-Cyclic-nucleotide 3′-phosphodiesterase (CNPase)-positive oligodendrocytes

4–6 slides after removing RNases with RNase Zap® and wiping down with absolute ethanol.

2. Roche Protector RNase Inhibitor 40 U/μl (Roche, IN). This is added to most aqueous solutions as a precautionary method to prevent degradation of RNA through RNase activity. Store at −20 °C and keep on ice while working with the product.
3. Liquid Blocker Super PAP pen (Daido Sangyo, Tokyo, Japan).

2.3 Rapid Immunohisto-chemistry Protocol for PV-Containing GABAergic Neuron Identification

1. 0.05 M TBS (Tris-buffered saline) solution pH 7.4 (Do not add a sodium azide preservative, as this interferes with down-stream processing).
2. 0.05 M TBS 0.2% Triton X-100 solution pH 7.4.
3. 0.05 M TBS 0.2% Triton X-100 1% BSA (Bovine serum albumin, Probumin® (Millipore, IL)). Aliquot 50 ml into a Falcon tube, stored at 4 °C.
4. Acetone.

5. 30% Hydrogen Peroxide. Store at 4–8 °C and only add to solutions just before needed (*see* Subheading 3).
6. Monoclonal anti-parvalbumin primary antibody (Sigma-Aldrich®, MO). After rehydrating the antibody, make 20 μl aliquots and store at −20 °C.
7. Peroxidase AffiniPure Donkey Anti-Mouse IgG secondary antibody (Jackson ImmunoResearch laboratories, PA). Use same storage protocol as primary antibody.
8. Roche Protector RNase Inhibitor 40 U/μl (Roche, IN).
9. NovaRED™ SK-4800 (Vector®, CA) stored at 4 °C and protected from light. When using this solution, wear appropriate personal protection equipment, such as a mask. The rinsate from this solution should also be disposed of with care, as the toxicity and carcinogenicity are unknown.
10. Molecular Sieves (EMD™, NJ). These sieves are added to the second 100% ethanol, in the dehydration series, and are to be changed every 4–6 slides.

2.4 Rapid Immunohistochemistry Protocol for 2′,3′-Cyclic-nucleotide 3′-phosphodiesterase (CNPase)-Containing Oligodendrocyte Identification

1. 0.05 M TBS (Tris-buffered saline) solution pH 7.4 (Do not add a sodium azide preservative, as this interferes with downstream processing).
2. 0.05 M TBS 0.2% Triton X-100 solution pH 7.4.
3. 0.05 M TBS 0.2% Triton X-100 1% BSA (Bovine Serum Albumin, Probumin® (Millipore, IL)). Aliquot 50 ml into a Falcon tube, stored at 4 °C.
4. Acetone.
5. 30% Hydrogen Peroxide. Store at 4–8 °C and only add to solutions just before needed (*see* Subheading 3).
6. Monoclonal anti-CNPase primary antibody (Mouse, EMD Millipore®). Make 4 μl aliquots and store at −20 °C.
7. Peroxidase AffiniPure Donkey Anti-Mouse IgG secondary antibody (Jackson ImmunoResearch laboratories, PA). Use same storage protocol as primary antibody.
8. Roche Protector RNase Inhibitor 40 U/μl (Roche, IN).
9. NovaRED™ SK-4800 (Vector®, CA) stored at 4 °C and protected from light. When using this solution, wear appropriate personal protection equipment, such as a mask. The rinsate from this solution should also be disposed of with care, as the toxicity and carcinogenicity are unknown.
10. Molecular Sieves (EMD™, NJ). These sieves are added to the second 100% ethanol, in the dehydration series, and are to be changed every 4–6 slides.

2.5 Laser (Capture) Microdissection: LMD or L(C)M

1. LEICA AS Laser Microdissection (LMD) apparatus fitted with 10×, 20×, and 40× magnification lenses used with Eppendorf thin-walled reaction tubes with domed cap for cell collection.
2. Arcturus^{XT}™ Laser-Capture Microdissection (LCM) System (MDS Analytical Technologies, CA) fitted with 10×, 20×, and 40× magnification lenses. Be sure to install the LMD or LCM equipment in a humidity and temperature-controlled environment, with humidity set below 40%, and kept at standard room temperature.
3. The Arcturus^{XT}™ is used with CapSure® HS LCM Caps (MDS Analytical Technologies, CA), which should be kept in a dark place until needed, and GeneAmp® thin-walled reaction tube with domed cap (Applied Biosystems, CA). Only the product category N8010611 fits perfectly around the HS cap to prevent any leakage.

2.6 RNA Extraction and Downstream Processing of Neurons after LCM or LMD

1. mirVANA™ miRNA Isolation Kit (Ambion, Austin, TX) or PicoPure® RNA Isolation kit (MDS Analytical Technologies, CA), used with the RNase-Free DNase Set (Qiagen, CA).
2. RiboAmp®HS^{PLUS} with Turbo Labeling™ Biotin (MDS Analytical Technologies, CA), used with SuperScript™ III Reverse Transcriptase (Invitrogen, CA). When performing this protocol, use 0.5 ml tubes correlating with a 0.5 ml plate in the thermal cycler. This will increase your mRNA yield significantly.

3 Methods

In this protocol, the LCM method includes a staining step: either Nissl or IHC, and is specifically tailored for nitrogen-vapor flash-frozen human postmortem brain tissue. However, either staining protocols can also be combined with LMD. The parameters such as cryostat temperature, section thickness, and LCM laser specifications will vary according to the type of tissue used and the fixation method, and should therefore be adjusted accordingly. For the IHC protocol, we noted in previous time study experiments that RNA in aqueous solutions started to degrade after 1.5 h. For this reason, our IHC protocol should be completed within 1 h. We have also added RNase inhibitors to all solutions in both staining protocols, to further prolong RNA viability. We therefore recommend that the LCM/LMD capture period should follow the staining protocol directly and should also not exceed 1 h. For our LMD method, no IHC is involved since DA neurons are neuromelanin positive and can readily be detected by light microscopy.

In general, when working with RNA, it is important to eliminate any contaminating RNase activity and surfaces should be

cleaned with RNase Zap® and wiped down with absolute ethanol. Using plasticware that is nucleic acid-free and changing your gloves regularly (especially after touching areas that are not RNase-free) will help reduce the risk of RNase contamination.

3.1 Tissue Preparation

1. Remove possible RNase-contamination on glass slide with RNase Zap®, followed by wiping it down with absolute ethanol.
2. Remove the tissue block stored in the −80 °C freezer and place it in a container with dry ice for transportation. Place a microslide box, previously treated with RNase Zap® and ethanol, in the same container. Keep the samples continuously on dry ice.
3. Adjust the cryostat temperature to −17 °C (*see* **Note 1**), install a new sectioning blade, and wipe the interior and sectioning blade with 100% ethanol with perpendicular motion away from the razor edge. Set the slice thickness to 7–8 μm (*see* **Note 2**). Do not open cryostat cover more than halfway to avoid moisture entering the cryostat.
4. After adhering the tissue block to the platform, let the block acclimate to the cryostat environment (temperature) for approximately 10–15 min.
5. Mount the tissue block to the specimen holder with as little "Optimum Cutting Temperature" (OCT) as possible, only touching the bottom of the specimen. Avoid covering the specimen with OCT along the sides. Only sections without OCT can be mounted for LCM.
6. Place specimen holder with mounted tissue such that the face of the tissue block is aligned with the blade's edge and the thinnest part of the tissue is cut first. Bring the stage/block closer to the blade—enough to better determine how to angle the block in relation to the blade. Adjust the block as necessary. Once it appears aligned from all angles, begin cutting at 7–8 μm until the tissue reaches the blade and it cuts through the tissue. Based on where/how the blade cuts (i.e., the angle of intersection with the tissue), adjust the block such that the blade will cut the surface of the tissue evenly to produce mountable sections for LCM/LMD.
7. Use a brush to wipe away frost on the blade and stage, and pieces of tissue that cannot be mounted. Always wipe brush away from the razor edge (never along or against it!).
8. After acquiring a suitable section, adhere it to the plain glass (LCM) or the Leica Frame Slide (LMD) toward the center of the slide at room temperature and place immediately thereafter inside the cryostat or into microslide box on dry ice (*see* **Note 3**). Do not allow slide to dry at room temperature.

9. Acquire another suitably smooth section (*see* **Note 4**), adhere it also toward the center of the same slide, and place slide into the micro slide box on dry ice. If cutting more than one specimen, use a new disposable microtome blade for each one. In addition, wipe down knife holder and stage with 100% ethanol in between each specimen to avoid cross-contamination.
10. Cut enough sections for your entire experiment. In our case, four sections (or two slides) per case were sufficient to capture between 300 and 500 neurons. When you are finished sectioning, place the micro slide box containing the sections into the −80 °C freezer until needed for staining and/or LCM or LMD.

3.2 Morphological Identification of Pyramidal Neurons with a Nissl Stain Procedure[1]

1. Prepare the ethanol dehydration series (2 × 75%, 2 × nuclease-free water, 95%, 100%), adding 25 ml of each ethanol into the appropriate jars.
2. Add molecular sieves to the 100% ethanol jar and the xylene jar under the fume hood.
3. Place the first 75% ethanol jar into the −20 °C freezer and all other jars on ice in an ice bucket.
4. Add 4 μl of RNase inhibitor to 400 μl of the HistoGene® stain in a 1.5 ml microcentrifuge tube (RNase-free) and place the tube on ice until needed.
5. Switch on the water bath and set to 42 °C and place a rack containing a 50 ml Falcon tube in the water bath.
6. Remove two slides with two sections each from the −80 °C freezer and defrost on a Kimwipe for 30 s or until the corners of the slides have defrosted.
7. With forceps, transfer the slides into the 75% ethanol jar that was originally placed in the −20 °C freezer for 30 s.
8. After 30 s in the second jar containing nuclease-free water, place the slides on a Kimwipe.
9. Circle the sections on the slide with a PAP pen (*see* **Note 5**) and aliquot 90 μl of the HistoGene® stain with RNase Inhibitor per section. Incubate at room temperature for 20 s.
10. Proceed through the dehydration series (nuclease-free water, 75%, 95%) until the final 100% ethanol dehydration step, leaving the slides in the final 100% ethanol jar containing the molecular sieves for 3 min (*see* **Note 6**).

[1] Protocol originally published in *JoVE*: *Pietersen CY, Lim MP, Woo TUW (2009)* [25]. *Obtaining High Quality RNA from Single Cell Populations in Human Postmortem Brain Tissue. JoVE. http://www.jove.com/index/details.stp?id=1444, doi: 10.3791/1444.*

11. Proceed to the fume hood and place the slides in the xylene jar containing the molecular sieves for 5 min, thereafter air-drying under the fume-hood for 5 min (*see* **Note 7**). Proceed immediately with LCM, capturing approximately 500 cells.

3.3 Rapid Immunohistochemistry Protocol for the Identification of PV-Containing GABAergic Neurons

1. Prepare solutions necessary for protocol beforehand: 0.05 M TBS, 0.05 M TBS with 0.2% Triton X-100 solution, 0.05 M TBS with 0.2% Triton X-100 and BSA solution.
2. RNase Zap® and rinse with nuclease-free water all containers, including wash bottles and slide jars that will contain solutions.
3. Fill one wash bottle with TBS solution and another with nuclease-free water. Fill a zapped slide jar with the Triton X solution (refresh this solution after each use).
4. In a 1.5 ml microcentrifuge tube, aliquot 990 μl TBS solution. Cap and leave on the bench at room temperature.
5. In two 0.5 ml PCR tubes, aliquot 174 μl of the TBS/Triton X-100/BSA solution in each. Defrost one aliquot of both the primary and secondary antibodies and add 20 μl of the primary antibody to one 0.5 ml PCR tube and 20 μl of the secondary to the other 0.5 ml PCR tube containing the TBS/Triton X-100/BSA solution. Place these two PCR tubes on 4 °C until needed.
6. Add 5 ml of nuclease-free water to a new 50 ml Falcon tube and place in rack on lab bench.
7. Prepare the ethanol dehydration series (50%, 75%, 95%, 2 × 100%), adding 25 ml of each ethanol into the appropriate jars. Add molecular sieves to the second 100% ethanol jar and a glass slide jar filled with xylene under the fume hood.
8. Switch on the water bath, set to 42 °C, and place a rack containing another 50 ml Falcon tube in the water bath.
9. Remove two glass slides with two sections each from the −80 °C freezer and defrost on a Kimwipe for 30 s or until the corners of the slides have defrosted.
10. Using forceps, place the slightly defrosted slides into a glass dish filled 3/4 with acetone (or just enough to cover the slides). Cover the dish, and incubate for 4 min to fix the tissue. This step should take place under a fume hood.
11. During this incubation time, prepare the endogenous enzyme block solution by adding 10 μl 30% hydrogen peroxide to the previously aliquotted TBS solution in the 1.5 ml microcentrifuge tube immediately before needed.
12. Remove the slides and rinse with TBS from the wash bottle, being careful not to place a direct stream onto the sections. Thereafter, rinse with TBS/Triton X-100 solution by dunking

slide into the previously prepared slide jar a few times. Rinse again with TBS from wash bottle. Repeat for the other slide.

13. With a Kimwipe, remove excess liquid around the sections on the slide, being careful not to touch the section itself. The excess liquid could cause dilution of the antibody in subsequent steps. Place the slides on an even surface, e.g., Kimwipe or the edge of an upside-down pipette tip container lid.
14. Pipette endogenous enzyme block onto slides—just enough to cover sections sufficiently (*see* **Note 8**)—and incubate for 5 min.
15. During this incubation period, add 6 μl of RNase Inhibitor to the primary antibody aliquot and TBS/Triton X/BSA solution in the 0.5 ml PCR tube from the fridge, just prior to use.
16. Rinse the slides gently with nuclease-free water from wash bottle, again not aiming the stream directly onto the sections themselves. Wipe off excess liquid as before and place on leveled surface.
17. Pipette 95 μl of the primary antibody solution onto the beginning of the slide. Gently place a glass coverslip, with its edge touching the antibody solution on the slide. Slowly lower the coverslip onto the slide, distributing the liquid evenly over both the sections on the slide, and avoid any bubble formation. Repeat for the other slide and incubate for 7 min.
18. During this incubation time, prepare the secondary antibody by adding 6 μl of RNase Inhibitor to the antibody aliquot and TBS/Triton X-100/BSA solution in the 0.5 ml PCR tube previously stored in the fridge.
19. After 7 min, rinse the slides as described in **step 12** and remove excess liquid (*see* **Note 9**).
20. Apply the secondary antibody following the method described for the primary antibody in **step 17** and incubate for 7 min.
21. During this incubation period, prepare the substrate chromogen (NovaRED) by following the instructions from the kit: Add 3 drops of reagent 1 to the previously prepared 5 ml nuclease-free water in the Falcon tube and mix, add 2 drops of reagent 2 and mix, add 2 drops of reagent 3 and mix. Only add hydrogen peroxide (2 drops) to the solution just prior to use for maximum results.
22. Place aluminum foil into a disused pipette container lid, and curve aluminum foil to form sides (will look like a dish). Place Kimwipes in the middle of the "dish" and wet slightly with nuclease-free water.

23. Rinse the slides as noted in **step 12** and remove excess liquid. Place the slides into aluminum foil "dish" and lay as flat as possible.
24. Pipette chromagen substrate solution onto the slides, making sure to cover the sections. Use more than necessary to make sure that the sections do not dry out. Incubate for 12 min.
25. Gently rinse the slides with the wash bottle containing nuclease-free water, again not directing the flow directly onto the sections, while collecting the rinsate into the aluminum foil "dish" and dispose appropriately.
26. Proceed with the dehydration series, by incubating the slides in each ethanol solution for 30 s beginning at 50%, and proceeding through to 100% ethanol. Leave the slides in the final 100% ethanol jar containing the molecular sieves for 3 min (*see* **Note 10**).
27. Proceed to the fume hood and place the slides in the xylene jar containing the molecular sieves for 2 min. Air-dry on a Kimwipe under the fume hood for 5 min. Proceed immediately with LCM, capturing approximately 350 cells.

3.4 Rapid Immunohistochemistry Protocol for CNPase-Containing Oligodendrocyte Identification

1. Prepare solutions necessary for protocol beforehand: 0.05 M TBS, 0.05 M TBS with 0.2% Triton X-100 solution, 0.05 M TBS with 0.2% Triton X-100, and BSA solution.
2. RNase Zap® and rinse with nuclease-free water all containers, including wash bottles and slide jars that will contain solutions.
3. Fill one wash bottle with TBS solution and another with nuclease-free water. Fill a zapped slide jar with the Triton X solution (refresh this solution after each use).
4. In a 1.5 ml microcentrifuge tube, aliquot 990 μl TBS solution. Cap and leave on the bench at room temperature.
5. In two 0.5 ml PCR tubes, aliquot 174 μl of the TBS/Triton X-100/BSA solution to one and 190 μl into the other. Defrost one aliquot of both the primary and secondary antibodies and add 4 μl of the primary antibody to the 0.5 ml PCR tube containing 190 μl of the TBS/Triton X-100/BSA solution and 20 μl of the secondary to the other 0.5 ml PCR tube. Place these two PCR tubes on 4 °C until needed.
6. Add 5 ml of nuclease-free water to a new 50 ml Falcon tube and place in rack on lab bench.
7. Prepare the ethanol dehydration series (50%, 75%, 95%, 2 × 100%), adding 25 ml of each ethanol into the appropriate jars. Add molecular sieves to the second 100% ethanol jar and a glass slide jar filled with xylene under the fume hood.

8. Switch on the water bath, set to 42 °C, and place a rack containing another 50 ml Falcon tube in the water bath.
9. Remove two glass slides with two sections each from the −80 °C freezer and defrost on a Kimwipe for 30 s or until the corners of the slides have defrosted.
10. Using forceps, place the slightly defrosted slides into a glass dish filled 3/4 with acetone (or just enough to cover the slides). Cover the dish, and incubate for 4 min to fix the tissue. This step should take place under a fume hood.
11. During this incubation time, prepare the endogenous enzyme block solution by adding 10 µl 30% hydrogen peroxide to the previously aliquotted TBS solution in the 1.5 ml microcentrifuge tube immediately before needed.
12. Remove the slides and rinse with TBS from the wash bottle, being careful not to place a direct stream onto the sections. Thereafter, rinse with TBS/Triton X-100 solution by dunking slide into previously prepared slide jar a few times. Rinse again with TBS from wash bottle. Repeat for the other slide.
13. With a Kimwipe, remove excess liquid around the sections on the slide, being careful not to touch the section itself. The excess liquid could cause dilution of the antibody in subsequent steps. Place the slides on an even surface, e.g., Kimwipe or the edge of an upside-down pipette tip container lid.
14. Pipette endogenous enzyme block onto slides—just enough to cover sections sufficiently (*see* **Note 8**)—and incubate for 5 min.
15. During this incubation period, add 6 µl of RNase Inhibitor to the primary antibody aliquot and TBS/Triton X/BSA solution in the 0.5 ml PCR tube from the fridge, just prior to use.
16. Rinse the slides gently with nuclease-free water from wash bottle, again not aiming the stream directly onto the sections themselves. Wipe off excess liquid as before and place on leveled surface.
17. Pipette 95 µl of the primary antibody solution onto the beginning of the slide. Gently place a glass coverslip, with its edge touching the antibody solution on the slide. Slowly lower the coverslip onto the slide, distributing the liquid evenly over both the sections on the slide, and avoid any bubble formation. Repeat for the other slide and incubate for 7 min.
18. During this incubation time, prepare the secondary antibody by adding 6 µl of RNase Inhibitor to the antibody aliquot and TBS/Triton X-100/BSA solution in the 0.5 ml PCR tube previously stored in the fridge.

19. After 7 min, rinse the slides as described in **step 12** and remove excess liquid (*see* **Note 9**).
20. Apply the secondary antibody following the method described for the primary antibody in **step 17** and incubate for 7 min.
21. During this incubation period, prepare the substrate chromogen (NovaRED) by following the instructions from the kit: Add 3 drops of reagent 1 to the previously prepared 5 ml nuclease-free water in the Falcon tube and mix, add 2 drops of reagent 2 and mix, add 2 drops of reagent 3 and mix. Only add hydrogen peroxide (2 drops) to solution just prior to use for maximum results.
22. Place aluminum foil into a disused pipette container lid, and curve aluminum foil to form sides (will look like a dish). Place Kimwipes in the middle of the "dish" and wet slightly with nuclease-free water.
23. Rinse the slides as noted in **step 12** and remove excess liquid. Place the slides into aluminum foil "dish" and lay as flat as possible.
24. Pipette chromagen substrate solution onto the slides, making sure to cover the sections. Use more than necessary to make sure that the sections do not dry out. Incubate for 12 min.
25. Gently rinse the slides with the wash bottle containing nuclease-free water, again not directing the flow directly onto the sections, while collecting the rinsate into the aluminum foil "dish" and dispose appropriately.
26. Proceed with the dehydration series, by incubating the slides in each ethanol solution for 30 s beginning at 50%, and proceeding through to 100% ethanol. Leave the slides in the final 100% ethanol jar containing the molecular sieves for 3 min (*see* **Note 10**).
27. Proceed to the fume hood and place the slides in the xylene jar containing the molecular sieves for 2 min. Air-dry on a Kimwipe under the fume hood until dry. Proceed immediately with LCM, capturing approximately 800 cells.

3.5 Preparation of Tissue Slides for LMD of Dopamine Neurons

Since midbrain dopaminergic neurons are neuromelanin positive, they are readily detectable in light microscopy (Fig. 1). Therefore, no additional staining steps are required. Prior to LMD the sectioned slides (Subheading 3.1) are dehydrated as described in Subheading 3.2, **steps 10** and **11**.

1. Load the slides and caps onto the Arcturus XT™ apparatus. Use the CapSure™ HS caps, but keep the program setting on Macro (*see* **Note 11**). Click on the box "Load with overview" to obtain an overview photo of each slide.

3.6 Laser Capture Microdissection of Pyramidal and PV-Containing GABAergic Neurons (See Footnote 1) and Oligodendrocytes

2. Adjust the brightness/focus at 2× magnification, in order to determine which section would be optimal for laser-capture. Avoid tissue sections with excessive folding, but rather choose sections that are intact, smooth, and stained well, especially near the region of interest.
3. Place a cap over the general area where capturing. Still at the 2× magnification, make sure that the cap rails do not rest on any folds, as this will tilt the cap.
4. Next, confirm the location of the IR laser spot manually at the 40× magnification. If the spot (red beam) does not correlate with the center of the blue cross, this can be adjusted by right-clicking on the spot and selecting "located IR spot."
5. Save the position of the cap by clicking on the plus sign at the position function. This way, if for any reason the cap must be removed and put back on the same location, the cap will always return to precisely the same spot that it was adjusted to before.
6. Enter these values into the control box: 70 into power and 16 into duration for neurons and 90/10 for oligodendrocytes. Unclick the "auto move stage" option, and make sure that the right size symbol correlates with the symbol on the panel to the right.
7. Select the capture circle option (bottom right) to select a cell that you want to test capture, and then activate the laser by clicking on "test IR spot."
8. The spot made by the laser should have a crisp dark ring around the object captured. If this ring is too light, the cell was not captured. If there is a dark spot in the middle of the dark ring, then the laser strength/duration is too great, and it might have a negative effect on the RNA present in the captured cell. Make sure that the ring is big enough to encompass the cell, but small enough that it does not include unwanted tissue or other cells (*see* **Note 12**).
9. Repeat the process on different parts of the tissue within the layer that you wish to capture, to check that the spot size does not differ depending on the location. Adjust laser power and duration accordingly.
10. Once the laser spot has been adjusted, identify pyramidal/PV GABAergic neurons and oligodendrocytes for capture (*see* **Note 13**). We identify approximately 350–500 neurons and 800 oligodendrocytes per sample, which results in approximately 400–800 pg of total RNA per sample (*see* **Note 14**).
11. Once all the cells have been captured, move the cap to a different part of the slide that does not contain a section. Go back to where the cells were captured and make sure that at least 80% of the neurons were removed (*see* **Note 15**). If not,

do not try to recapture the same cells, but locate the area where most of the cells were captured, and capture more cells in the same area. Move the cap to the QC station.

12. Place the cap into the 0.5 ml microcentrifuge tube from Applied Biosystems containing 50 μl of extraction buffer (PicoPure® RNA Isolation kit). The cap has been designed to fit perfectly into this specific tube to prevent buffer from leaking.
13. Turn the assembly upside-down, making sure that extraction buffer covers the entire cap, and place it at the bottom of the 50 ml Falcon tube already present in the water bath set at 42 °C. The cells are incubated for 30 min in order to remove the tissue from the cap.
14. Thereafter, centrifuge the tube and cap assembly for 2 min at 800 × *g*. Remove the cap and store the remaining cell extract at −80 °C until RNA extraction. As an extra precaution, you can re-examine the cap at the QC station on the laser-capturing apparatus to ensure that all cells have been removed from the cap itself.

3.7 LMD of Dopamine Neurons

1. LMD is performed according to the manufacturer's instructions. We use a LEICA AS Laser Microdissection (LMD) apparatus with manual stage.
2. After setting up the hard- and software of the instrument place the FrameSlides with the tissue facing down and insert an Eppendorf thin-walled reaction tube with the domed cap in the tray and secure the tube in position.
3. Select the requested tube position and program, calibrate the laser, and adjust the laser to "line" setting to outline each individual cell (we capture at 40× magnification). Then complete the capture in standard mode. While capturing use the "Move and Cut" mode to remove cells that are caught on the static of the slide. Click on the cell until it appears to have fallen (*see* **Note 16**).
4. After capturing click "no cap." Unload and remove tray and slide and with the tube still in tray, add 50 μl lysis buffer (Lysis/Binding Buffer mirVana miRNA Ambion Isolation Kit) to the cap (*see* **Note 17**). Close the tube and remove from tray, store the tube in −80 °C.

3.8 RNA Extraction and Downstream Processing of Cells after LCM or LMD

1. RNA of LCM Pyramidal/PV-containing GABAergic neurons was isolated using the PicoPure® kit and LMD DA neurons with the mirVANA™ miRNA Isolation Kit. During RNA isolation with the PicoPure® kit, follow the appendix protocol for DNase treatment of the sample, as the elimination of genomic DNA is critical for accurate downstream applications such as qRT-PCR.

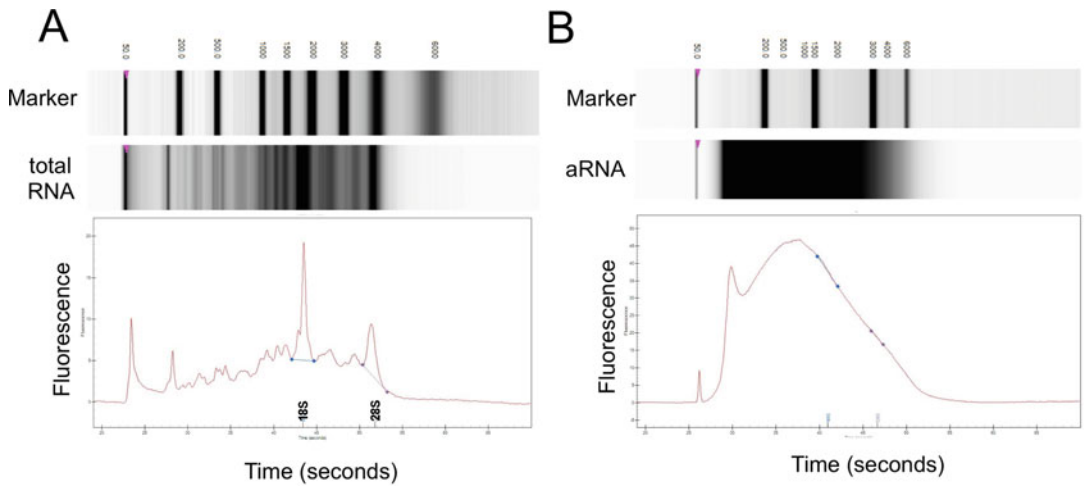

Fig. 2 Isolation of high-quality total RNA (**a**) and generation of aRNA (**b**) from ~1000 pyramidal neurons using the PicoPure RNA isolation kit. Samples were analyzed on an Agilent 2100 Bioanalyzer. Shown are gel images (top) and electropherograms (bottom). RIN = 7.2

2. Amplify the mRNA (two rounds) obtained from pyramidal/PV-containing GABAergic neurons with the RiboAmp®HS-PLUS kit and provided protocol. During the second strand cDNA synthesis, our lab has made one slight modification to the manufacturer's protocol. When the sample has been transferred from the tube to the purification column, centrifuge the tube originally containing the sample and transfer the extra microliter obtained after centrifugation into the purification column. Repeat this process during the second round of amplification (*see* **Note 18**).
3. To assess (m)RNA quality, the A_{260}/A_{280} ratio can be measured with a NanoDrop spectrophotometer and the samples can be analyzed by an Agilent Bioanalyzer (Fig. 2). If the amplified mRNA from the LCM neurons is used for microarray analysis, you should run an Experion StdSens LabChip® to ensure that the transcript length exceeds 600 nucleotides required for microarray hybridization (*see* **Note 19**).
4. RNA obtained from LMD material was used for microarrays [9, 10, 13] and miRNA profiling with a TaqMan® quantitative Real-Time PCR based on the TaqMan® Human MicroRNA A Arrays (Applied Biosystems, CA) [14, 15].

3.9 Downstream Applications

After viable RNA is achieved with any of the above methodologies, it can be used to evaluate the expression profiles of cellular populations via a variety of technologies available today. These include microarray, whole genome sequencing (RNA-Seq), or polymerase chain reaction (PCR) technologies to determine the complete gene (mRNA and ncRNA) expression profiles of the cell types of interest.

For example, we have used the resulting RNA from the LMD protocol for microarray and micro (mi)RNA profiling of postmortem dopaminergic neurons in Parkinson's disease subjects [9, 10, 14, 15], and this method was also used in combination with the Nissl staining technology to determine the expression profiles of hippocampal GABAergic neurons [13]. We have also analyzed the microarray and miRNA data obtained from the pyramidal and parvalbumin neurons, as well as oligodendrocytes in postmortem schizophrenia subjects and controls to discover altered underlying molecular pathways in this patient population [6–8]. In addition to our studies, there have been a number of other reports describing cell type-specific gene expression profiles from postmortem human brains to study the molecular properties of neuropsychiatric diseases, such as Alzheimer's disease [16–18], amyotrophic lateral sclerosis [19], major depression [20], multiple sclerosis [21], and schizophrenia [22], or to assess the cell type-specific characteristics of the healthy human brain [23–25].

4 Notes

1. As temperature can also influence tissue smoothness, try to keep the temperature above −20 °C. Temperatures lower than this can result in tissue cracking.
2. You must be able to visualize the structure you wish to capture after staining. In our case, neurons are identified via a stain. If you cut the sections too thin, you might not be able to adequately visualize these cells. If the section is too thick, cells designated for capture will not fully adhere to the cap, and part of the cell will remain on the slide, therefore, decreasing RNA yield.
3. The tissue moves between different temperature states during sectioning between the dry ice (approximately −78 °C), cryostat, room temperature, and eventually the −80 °C freezer environments. In order to reduce the temperature gradient, do not raise the temperature of the cryostat to more than −15 °C, as it could affect RNA integrity.
4. You must be able to cut the tissue into smooth sections without folding. In order to accurately capture the designated area, smooth sections are key. Too thick or too thin sections could result in folding or tearing respectively.
5. The PAP pen helps to concentrate the staining solution onto the section. However, with the plain glass slides, it is not always necessary to use it, as the staining solution appears to cling only to the section itself.

6. The molecular sieves draw out any excess moisture that might be present in the absolute ethanol and xylene solutions. This helps to dehydrate the sections completely, which is a necessary component for LCM.
7. Check that the sections are completely dry before proceeding with LCM. If you are still not able to capture cells after dehydration and air-drying, repeat the absolute ethanol, xylene, and air-drying steps to ensure proper dehydration.
8. We have opted not to use a humid chamber during the IHC protocol, as it is not necessary for such short incubation times. However, whenever applying a solution without a coverslip onto the sections, apply enough to ensure that the sections do not dry out during the incubation time.
9. The glass coverslips should come off while rinsing. Do not manually try to remove the coverslips, as this will cause some of the tissue section to scrape off. Adding enough liquid between the slide and coverslip, either primary or secondary antibody, will ensure a smooth coverslip removal.
10. We extended the duration of the final dehydration of the sections in 100% ethanol from 30 s to 3 min, and also added molecular sieves to the jar to ensure sufficient dehydration of the tissue, to obtain adequate tissue lift and to preserve RNA integrity. Molecular sieves are also added to the xylene solution for this reason.
11. As the HS cap is made for capturing single cells, it only has a small area designated for capture, which can limit the section area from which you would like to capture. To alleviate this issue, keep the settings on the Arcturus^{XT}™ software on Macro, while using the HS cap, in order to capture cells from a larger section area.
12. The spot size should be specific to your cell size, i.e., not too big that it is nonspecific and small enough to be as specific as possible. At the same time, the laser strength should still be strong enough to be able to capture the cell. Conversely, if the laser strength is too high, the spot size will enlarge and might not be specific to the cell of interest.
13. Do not include cells that have other cell-types directly next to it, as these may also be captured and you will no longer have a homogeneous cell population. Also try to limit the amount of surrounding tissue you capture with the cell of interest by decreasing the spot size, while still ensuring adequate capture of that cell.
14. We also usually only use one section in order to reduce the amount of capture time, as the cap has to be readjusted for each section. Although, if you have not succeeded in capturing all cells in the allotted time, you can repeat the stain and capture from different sections from the same case.

15. Sometimes, during humid weather or particularly aqueous procedures, such as IHC, the tissue is not completely dry. In this case, and if additional dehydration does not help, use the Macro cap as it is in direct contact with the tissue, and therefore adheres to the cells more easily. Keep in mind, however, that this cap often picks up unwanted tissue "debris" for the same reason. In this case, use the prep strips that are provided, which remove the debris from the surface of the section prior to microdissection.
16. We found that larger pieces of tissue more likely cling to the slide via static electricity, so avoid capturing more than one cell at a time. When cells do reattach to the slide, they are most easily removed by directing the laser toward the center of the cell.
17. We found that lysis buffer dries out quickly, so we recommend adding the buffer after capturing.
18. We recommend performing the amplification procedure using the 0.5 ml tubes provided, and subsequently, performing the thermal cycling with a corresponding 0.5 ml block to increase the RNA yield.
19. In our experience, the sensitivity of the NanDrop is insufficient to reliably detect RNA in the pg and lower ng range. In contrast, the Agilent 2100 Bioanalyzer provides a Nano or a Pico Kit, which is sufficient to measure such low RNA concentrations and should be the method of choice for quality assessment. In addition, RINs from LCM or LMD isolated neurons tend to be lower than from isolated whole tissue, but RINs are not necessarily a reliable measure of RNA integrity (*see* [2, 3] for further details).

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Chapter 12

The Isolation of Pure Populations of Neurons by Laser Capture Microdissection: Methods and Application in Neuroscience

Renée Morris and Prachi Mehta

Abstract

In mammals, the central nervous system (CNS) is constituted of various cellular elements, posing a challenge to isolating specific cell types to investigate their expression profile. As a result, tissue homogenization is not amenable to analyses of motor neurons profiling as these represent less than 10% of the total spinal cord cell population. One way to tackle the problem of tissue heterogeneity and obtain meaningful genomic, proteomic, and transcriptomic profiling is to use laser capture microdissection technology (LCM). In this chapter, we describe protocols for the capture of isolated populations of motor neurons from spinal cord tissue sections and for downstream transcriptomic analysis of motor neurons with RT-PCR. We have also included a protocol for the immunological confirmation that the captured neurons are indeed motor neurons. Although focused on spinal cord motor neurons, these protocols can be easily optimized for the isolation of any CNS neurons.

Key words Laser capture microdissection (LCM), Brain-derived neurotrophin factor (BDNF), Tropomyosin receptor kinase B (TrkB), High-quality RNA, Motor neurons, Spinal cord, Gene expression

1 Introduction

The mammalian central nervous system (CNS) is comprised of a number of functionally different populations of neurons that are organized into highly specific systems. For instance, brain centers specialized in sensory processing capture information regarding the nature of stimuli—whether visual, tactile, auditory, or olfactory—that are present in the environment. These modality-specific sensory areas decode the information and convey it to the motor regions of the brain and subsequently to the motor neurons lying in the ventral horn of the spinal cord. The axons of these motor neurons project their axons outside the CNS, mainly onto skeletal muscles and other effector organs. Although they play a central role in the execution of voluntary movement, the gene expression

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profile of motor neurons has yet to be satisfactorily characterized. The main reason for this knowledge gap resides in the fact that motor neurons, which represent less than 10% of the total spinal cord cell population, are not amenable to transcriptional profiling using conventional spinal cord homogenate.

Laser capture microdissection (LCM), first developed by Emmert-Buck and collaborators [1], has been successfully used to isolate specific populations of cells in the CNS for a variety of downstream gene expression analysis, successfully circumventing the issues associated with tissue heterogeneity [2–8]. In this book chapter, we describe a protocol developed in our laboratory that uses LCM to capture pure population of motor neurons and to isolate total RNA of high quality from these neurons [9]. Procedure for downstream RT-PCR analysis with primers for brain-derived neurotrophic factor (BDNF)—a growth factor widely expressed in the spinal cord- and its tropomyosin kinase B receptor (TrkB) is also described. A protocol for immunohistochemistry with an antibody raised against choline acetyltransferase (ChAT)—the enzyme responsible for the synthesis of the neurotransmitter acetylcholine and a marker for motor neurons is also described in this chapter. We believe that this LCM protocol can be adapted to any other types of cell in the CNS.

2 Materials

Prepare all solutions with room temperature double distilled or MilliQ water. Store the solutions at room temperature unless indicated otherwise. Ensure all the solutions are labeled properly.

2.1 Animal Perfusion and Tissue Collection

1. 0.1 M phosphate-buffered saline (PBS), pH 7.4: pour 800 ml of double distilled or MilliQ water into a 1000 ml Duran laboratory bottle. Add 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4 to the water. Adjust the pH to 7.4 with HCl. Add distilled water to a total volume of 1000 ml.
2. Paraformaldehyde solution in 0.1 M PBS: add 2 g of paraformaldehyde to 48 ml of 0.1 M PBS. Heat to dissolve. Add drops of 2 M NaOH until the solution clears. Add 50 ml of 0.1 M PBS and mix. Remove from heat and let the solution cool down. Adjust the pH from 7.2 to 7.4 (*see* **Note 1**).
3. RNAse-free diethylpyrocarbonate (DEPC)-treated water: fill a 1000 ml Duran laboratory bottle with 999 ml of MilliQ or double distilled water and add 1 ml of diethylpyrocarbonate. Incubate the water for 12 h at 37 °C. Autoclave DEPC-water for 15 min. Commercially available, ready to use ultra pure DEPC water can be used to reduce labor.

4. *RNAse-free microscope slides*: dip the microscope slides into undiluted RNAse away (Ambion) for a few seconds. Rince the slides twice in DEPC-treated water. Dry at 37 °C for at least 30 min. Store the slides in a clean slide box (*see* **Note 2)**.

2.2 Tissue Staining

1. RNAse-free 70% ethanol: mix 147.4 ml 95% ethanol with 52.6 ml DEPC-treated water.
2. 1% Azure B dye solution: place 0.3 g Azure B dye into a sterile 50 ml conical centrifuge tube. Add 30 ml of RNAse-free ethanol to the tube. Mix on a rocker overnight.

2.3 Immunohistochemistry

1. Permeabilization solution: add 1 ml Triton X-100 to 199 0.1 M PBS. Mix on a stirrer until Triton X-100 is completely dissolved.
2. Nonspecific staining blocking solution: add 10 ml normal donkey serum to 90 ml of permeabilization solution. Mix well.

3 Methods

3.1 Animal Perfusion

1. Euthanize the rats with a solution containing one part of Lethabarb (100 mg/kg, Virbac, Australia) and one part of physiological saline delivered intraperitonneally (*see* **Note 3**).
2. Once absence of corneal and toe-pinch response is ascertained, place the rat on its back on a dissecting board over a perfusion sink.
3. Lift the sternum and the skin covering it with a pair of large forceps with tooth grips.
4. With a pair of strong surgical scissors, make a skin incision from right below the sternum to both armpits.
5. Cut the diaphragm to expose the heart and deliver 0.1 ml heparin into the apex of the heart to prevent blood coagulation.
6. Cut out the apex of the heart and insert a cannula attached to a peristaltic pump into the aorta through left ventricle and immediately clamp the cannula in situ with a hemostat.
7. Make a small incision in the right atrium with a pair of fine scissors and immediately start the peristaltic pump. Perfuse with 0.1 M PB until the liquid flowing out of the atrium is nearly free of blood.
8. If appropriate, stop the peristaltic pump and switch the perfusate valve to a solution of 4% paraformaldehyde in 0.1 M PBS (*see* **Note 4**).
9. Run the pump for a few extra minutes until perfusion tremors are observable (*see* **Note 5**).

3.2 Tissue Dissection

1. Place the rats in prone position onto a dissection table and make an incision with a scalpel blade on the midline of the animals' back over the cervical spinal cord segment(s) of interest. Alternatively, the animal should be in supine position for lumbar spinal cord dissection (*see* **Note 6**).
2. Reflect the back muscles to expose the vertebral column.
3. Perform a laminectomy over the spinal cord segment(s) of interest with rongeurs to expose the spinal cord and dorsal roots.
4. With a new scalpel blade, dissect out the spinal cord tissue in blocks containing two segments, place them individually in small Corning culture dishes, and rinse the tissue for 10 s in RNase-free water.
5. Place the two-segment blocks of tissue at the bottom of a cryomold filled with OCT. Ensure that the specimens are lying flat at the bottom of the cryomold to ensure proper cutting.
6. Place the cryomolds in a shallow tray filled with 2-methyl butane prechilled in a bath of liquid nitrogen to fast-freeze the tissue.
7. Store the OCT-embedded frozen blocks at −80 °C until needed.

3.3 Sectioning and Staining the Spinal Cord Tissue

1. Cut the OCT-embedded blocks of tissue into longitudinal sections and mount the tissue directly onto RNAse-free microscope slides (*see* **Note 7**).
2. For staining, place four sterile 50 ml conical centrifuge tubes on a centrifuge tube rack (*see* **Note 8**).
3. Fill the first three tubes with 30 ml RNAse-free 70% ethanol.
4. The fourth tube should contain a1% solution of Azure B.
5. Take the slides of the −80 °C freezer and immediately place them on dry ice.
6. Thaw the slides one at a time by quickly holding them on a gloved palm and wipe off the moisture around the tissue and at the back of the slide with disposable wipers (Kimwipe, Sigma-Aldrich). Subsequently place the slide onto a bed of fresh silica gel desiccant for 40 s.
7. Immerse the slide into the first tube of ethanol for 40 s, then dip it up and down into the tube for an additional 40 s to remove the OCT around the tissue sections.
8. Drained the excess ethanol with disposable wipers. Repeat this process by immersing and dipping the slide up and down in the second tube of ethanol. Drain again with disposable wipers. Repeat this step once more if needed.

9. Immerse the slide into the tube containing the Azure B staining solution for 40 s and drain the excess stain.
10. Differentiate the tissue by immersing the slide into the third ethanol-filled tube. This step can be repeated if needed by immersing the slide into a new tube containing 30 ml RNAse-free 70% ethanol. If the staining is too pale, re-immerse the slide into the tube of Azure B solution and repeat the differentiation process.

3.4 Laser Capture Microdissection (LCM)

1. Turn on the LCM system and unload its microcentrifuge tube holder (*see* **Note 9**). Attach the cap of a 0.6 ml tube containing 30 μl guanidine thiocyanate lysis buffer (Sigma-Aldrich) to the holder to collect the samples.
2. Place the slide on the stage of the microscope and, with the 20× objective, delineate the contour of each neuron with the LCM software (*see* **Note 10**).
3. Turn the UV laser to dissect out and catapult the neurons into the cap of the microcentrifuge tube, after which remove the tube from the holder. Completely lyse the neurons by pipetting the lysis buffer up and down (*see* **Note 11**).
4. Close the microcentrifuge tube and centrifuge to collect the motor neuron-containing solution at the bottom of the tube. Freeze the sample onto dry ice and store at −80 °C (Fig. 1, *see* **Note 12**).

3.5 RNA Extraction and Determination of RNA Quantity and Quality

1. Extract the RNA with an RNeasy Micro Kit (Quiagen) according to the manufacturer's protocol for extracting RNA from LCM samples.
2. Reserve 1 μl RNA to determine RNA concentration with a NanoDrop instrument (Thermo Fisher Scientific) and 2 μl to establish RNA quality with a capillary electrophoresis microfluidics chip (Argilent 2100 Bioanalyzer) (*see* **Note 13**). Fast freeze the remaining RNA on dry ice and then store at −80 °C until needed (Fig. 2).

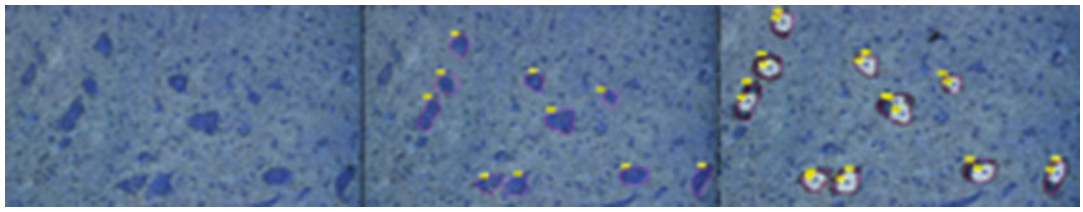

Fig. 1 Before-and-after images of a laser dissection of Azure B-stained motor neuron. (**a**) Image of spinal cord specimen with Azure B stained motor neurons. (**b**) The motor neurons have been outlined and assigned a number by the LCM software. (**c**) The same specimen than in A and B after the laser capture of the motor neurons, leaving small holes in the tissue where the motor neurons used to be located

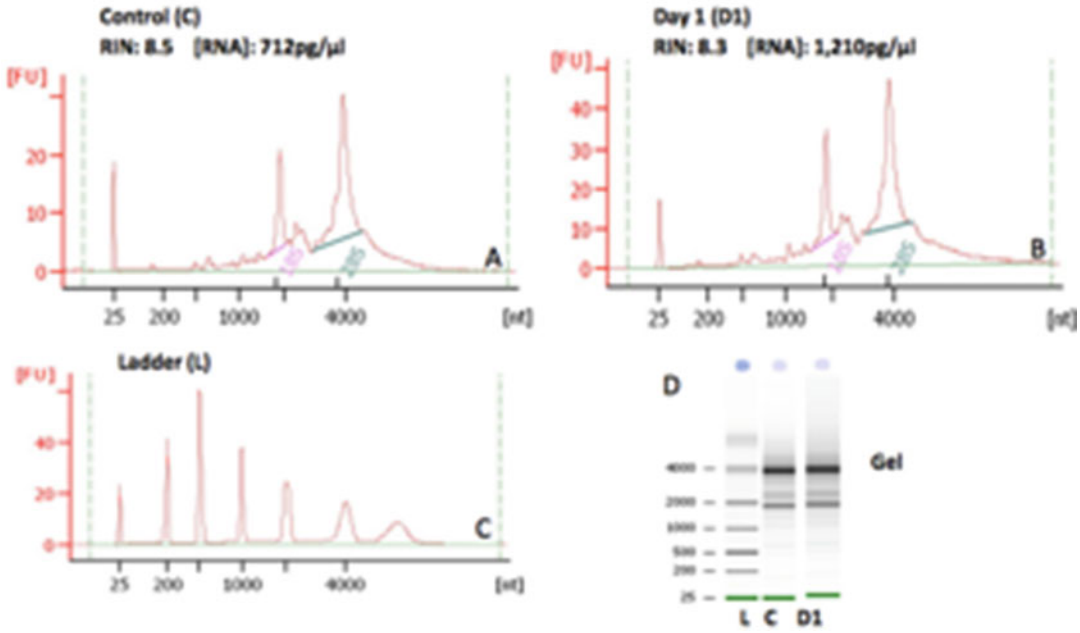

Fig. 2 Electrophoretic analysis of the integrity of RNA (RIN) from LCM samples. The electropherograms of the (**a**) control and (**b**) Day-1 samples as well as (**c**) the size standards and (**d**) the corresponding gel image for standard and samples

3.6 cDNA Synthesis

1. Synthesize the cDNA with SuperScript III First Strand Synthesis System (Thermo Fisher Scientific) according to the manufacturer's protocol. In a 0.6 ml microcentrifuge tube, mix 4 µl of isolated RNA, 1 µl of random hexonucleotides, 2 µl of 10 mM dNTP mix, and 3 µl DEPC-treated water (10 µl final volume). Incubate the reaction tube at 65 °C for 5 min and immediately chill the reaction sample on ice.
2. In a second tube, prepare the cDNA synthesis mix, i.e., mix 10× RT buffer, 25 mM $MgCl_2$, 0.1 M DTT, RNAse OUT and SuperScript III reverse transcriptase (10 µl final volume) and add the mixture to the first tube.
3. Incubate the reaction tube (20 µl final volume) at 25 °C for 10 min and then at 50 °C for 50 min. Terminate the reaction at 85 °C for 5 min and immediately chill on ice.
4. Add 1 µl of RNase H to the tube, mix and incubate at 37 °C for 20 min followed by 95 °C for 10 min. Store the cDNA at −20 °C until needed (*see* **Note 13**).

3.7 Polymerase Chain Reaction (PCR)

1. Mix together 10× PCR buffer, 10 mM dNTP mix, 50 mM $MgCl_2$, 10 µM of each primer, platinum Taq DNA Polymerase (Sigma-Aldrich) and DEPC-treated water to a final volume of 45 µl. Add 5 µl of cDNA to the above mix (total volume 50 µl).

Table 1
PCR primer sequences, product sizes and nucleotide reference numbers

Genes	Primer Sequence (5′ to 3′)	Product size	Nucleotide ref. No.
BDNF	Forward: CGAGACCAAGTGTAATCCCA Reverse: TCTATCCTTATGAACCGCCA	120	Bp 920–1075 of NM_001270630.1
TrkB	Forward: TCTCATTTTAGGCCGCTTTG Reverse: GGGTTTGAGGTGGGTGAAG	118	NM_012731.1
GAPDH	Forward: CCTGCACCACCAACTGCTTAGC Reverse: GAGTTGCTGTTGAAGTCACAG	417	X02331 (Gene Bank)/ NM_017008.4
B2M	Forward: CCGTGATCTTTCTGGTGCTT Reverse: TTTTGGGCTCCTTCAGAGTG	318	NM_012512

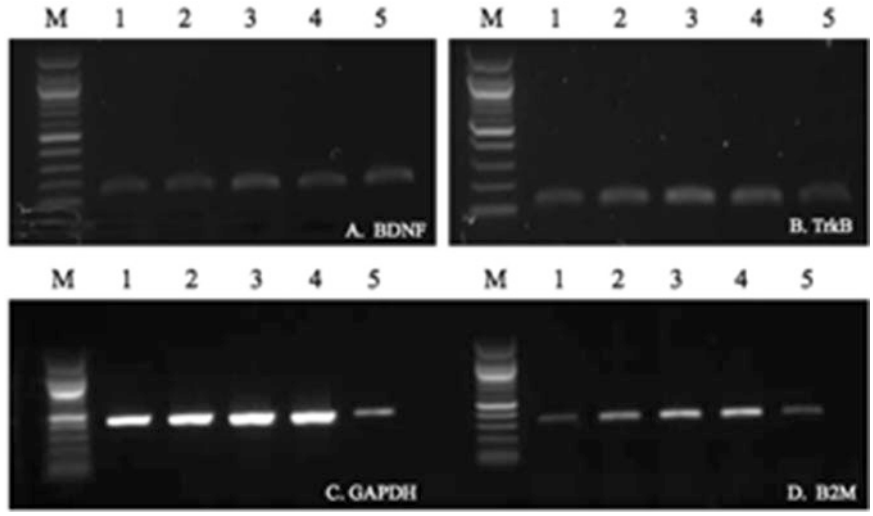

Fig. 3 RT-PCR of samples amplified for (**a**) BDNF, (**b**) TrkB, (**c**) GAPDH and (**d**) B2M genes. M: Molecular weight marker,1: C2–C3 motor neurons from control sample, 2: C4–C5 motor neurons from control sample, 3 = C2–C3 motor neurons from Day 1sample, 4 = C4–C5 motor neurons from Day 1 sample and 5 = spinal cord homogenate sample

2. Perform amplification using the "hot start" method with the following cycling profile: initial denaturation at 94 °C for 2 min, 35 cycles of denaturation at 94 °C for 30 s each, annealing at 50.1 °C for 30 s, and extension at 72 °C for 1 min. Perform a final extension at 72 °C for 5 min and cool the PCR products to 4 °C (Note: must be optimized).
3. Separate the PCR products electrophoretically on a 1% agarose gel and stain with SYBR safe (Thermo Fisher Scientific).
4. Primers used for amplification include BDNF, TrkB, and two reference genes GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) and B2M (Beta 2 microglobulin) (*see* Table 1 for the different gene sequences) (Fig. 3).

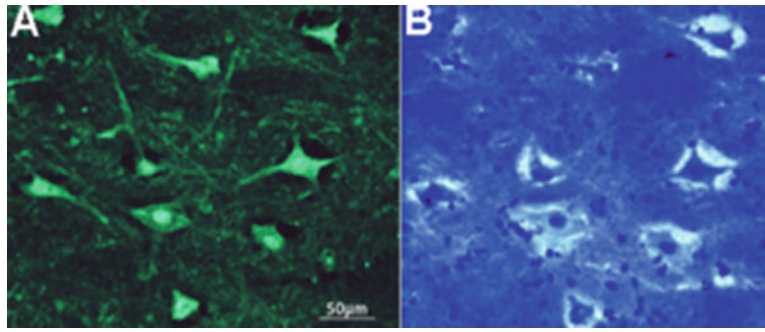

Fig. 4 Anti-ChAT and Azure B staining of motor neurons. (**a**) Photomicrographs of ventral horn motor neurons positive for anti-ChAT and (**b**) photomicrograph of the same spinal cord region showing that the motor neurons in (**a**) are also positively stained with Azure B. Reprinted with permission from [9]

3.8 Immunostaining

1. Retrieve microscope slides with spinal cord tissue from the −80 °C freezer, thaw and wipe off as much moisture as possible, taking care not to damage the tissue.
2. Wash the tissue in 0.1 M PBS (*see* **Note 14**) followed by a 15 min immersion in paraformaldehyde.
3. Wash the slides again in 0.1 M PBS and permeabilize the tissue for 15 min.
4. Block nonspecific staining for 30 min at 37 °C.
5. Dilute the goat anti ChAT primary antibody (Millipore AB144p) in 1:500 in 0.1 M PBS and incubate the tissue overnight at 4 °C.
6. Wash the thoroughly in 0.1PBS and incubate in secondary antibody Alexa Fluor 488 donkey anti-goat IgG (Thermo Fisher Scientific) diluted 1:1000 in 0.1 M PBS for 1 h.
7. Wash the slides thoroughly in 0.1PBS, allow to dry, and then image through the FITC channel (*see* **Note 15**).
8. Re-hydrate the tissue in 0.1 M PBS and stain with 1% Azure B dye solution for 2 min. Allow to dry and image with bright field microscope (Fig. 4).

4 Notes

1. Paraformaldehyde must be weighed and the solution prepared in a safety cabinet and goggles, lab coat and appropriate gloves must be worn as it is toxic and can cause severe eye and skin irritation. For safety reasons, we favor prilled formaldehyde (Sigma-Aldrich), the basic building block of paraformaldehyde, as a safer substitute to paraformaldehyde in a powder form. Neutral buffered formalin (Thermo Fisher Scientific) diluted

into a 2% solution in 0.1 M PBS is an even safer alternative (i.e., no need to heat the solution and correct the pH). In our hands, neutral buffered formalin preserves RNA integrity in a similar manner than paraformaldehyde.

2. Collecting tissue specimens by LCM for downstream RNA extraction can be costly if commercially available membrane-coated slides are used. We use standard microscope slides and decontaminate them with undiluted RNase AWAY. In our hands, this treatment preserves the RNA integrity as well as those above-mentioned expensive membrane-coated slides.
3. Due to the irritant properties of Lethabarb when delivered intraperitonneally, it is a requirement of the Animal Care and Ethics Committee of the University of New South Wales to dilute Lethabarb at least in half with physiological saline before delivering it into the abdominal cavity. Check the policies of your Institute in this regard.
4. We systematically tested whether the addition of paraformaldehyde in the perfusion protocol led to better RNS integrity than PBS alone and found no difference between PBS alone and PBS followed by paraformaldehyde (Mehta et al. [9]). Based on these findings, we currently omit the use of paraformaldehyde in our protocol.
5. The time required for animal perfusion and tissue collection must be as brief as possible to preserve the RNA integrity and should not take longer than 10–16 min to ensure high-quality RNA. A few rounds of practice with spare tissue are advisable to gain speed.
6. *See* reference 10 for the complete visual protocol we developed for the dissection of the cervical and lumbar parts of the spinal cord.
7. In several studies, the tissue is cut at 20 μm, leading to the generation of a high number of slides. Generating 50 μm-thick tissue sections (i.e., instead of thinner tissue sections) greatly diminishing the number of slides needed, therefore contributing to keep the costs of LCM low. In our hands, cutting thicker tissue sections does not affect the ability of the UV laser to catapult the neurons into the cap of the microcentrifuge tubes. It also protects the RNA integrity by exposing less histological material to possible contaminant. We also routinely mount as little as four spinal cord sections per slide to reduce the amount of time it takes to complete the LCM process, therefore minimizing RNA degradation during this step of the protocol.
8. The microscope slides are kept on dry ice and are moved into an antechamber adjacent to the microscopy room where the staining is performed one slide at a time. After the completion

of the staining steps, the slide is immediately moved to the microscopy room where it is placed onto the LCM stage.

9. We use a PALM Duoflex combi system (Carl Zeiss).
10. During the delineation step with the LCM software it is important to include the neurons' external membrane, especially if the RNA of interest is membrane-bonded.
11. The small volumes of guanidine thiocyanate lysis buffer can hold up to 2000 motor neurons collected from one spinal cord sample.
12. The process for one slide, from Azure B staining to the end of neuron collection, should be completed within 30 min to minimize RNA degradation. Here again, a few rounds of practice with spare tissue are advisable to gain speed.
13. The Argilent 2100 Bioanalyzer system includes an algorithm that assign an RNA Integrity Number (RIN) to each sample. A RIN represents a measurement of the quality of total RNA samples including their purity and concentration [11]. RIN scores vary from 1 to 10, and scores from 8 to 10 indicate highly preserved RNA. In the present protocol, RNA samples with RINs ≥ 8 are considered optimal for further downstream analysis and samples with RIN < 8 are discarded.
14. Whole spinal cord homogenate is used as a positive control whereas the negative control did not contain RNA.
15. The washes in 0.1 M PBS are best performed with agitation on a rocker.

Acknowledgement

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Chapter 13

Laser Capture Microdissection in Traumatic Brain Injury Research: Obtaining Hippocampal Subregions and Pools of Injured Neurons for Genomic Analyses

Deborah R. Boone, Harris A. Weisz, Stacy L. Sell, and Helen L. Hellmich

Abstract

The methods presented here are based on our laboratory's 15 years of experience using laser capture microdissection to obtain samples for the study of gene expression after traumatic brain injury (TBI) using a well-established rat model of experimental TBI. Here, we describe how to use the Arcturus^{XT} laser capture microdissection system to capture swaths of specific regions of the rat hippocampus as well as specific neuronal populations defined by Fluoro-Jade C staining. Staining with Fluoro-Jade C identifies a neuron that is in the process of degeneration. We have optimized our protocols for Fluoro-Jade C tissue staining and laser capture microdissection to maintain RNA integrity which is essential for a variety of downstream applications, such as microarray, PCR array, and quantitative real-time PCR analyses.

Key words Brain injury, Gene expression, Hippocampus, Single cell, Neuron, Fluoro-Jade C

1 Introduction

Since initially described [1], laser capture microdissection (LCM) methods have allowed researchers to obtain identified cells of interest from sections of complex, heterogeneous tissues that can be used for molecular analyses. Due to the remarkable complexity and heterogeneity of the mammalian brain with its hundreds to thousands of cell types [2], molecular analyses pose distinct challenges to neuroscientists. Laser capture techniques have provided a new platform for exploring brain function, and proven to be a boon for molecular neuroscience. We show here that LCM has been particularly useful for the study of the pathogenesis of traumatic brain injury (TBI).

Our laboratory has used LCM [3] to probe the molecular underpinnings of TBI in our well-established rat model of TBI [4] for over 15 years. We use The Arcturus^{XT} LCM system with its UV cutting and IR lasers which allow precise capture of distinct

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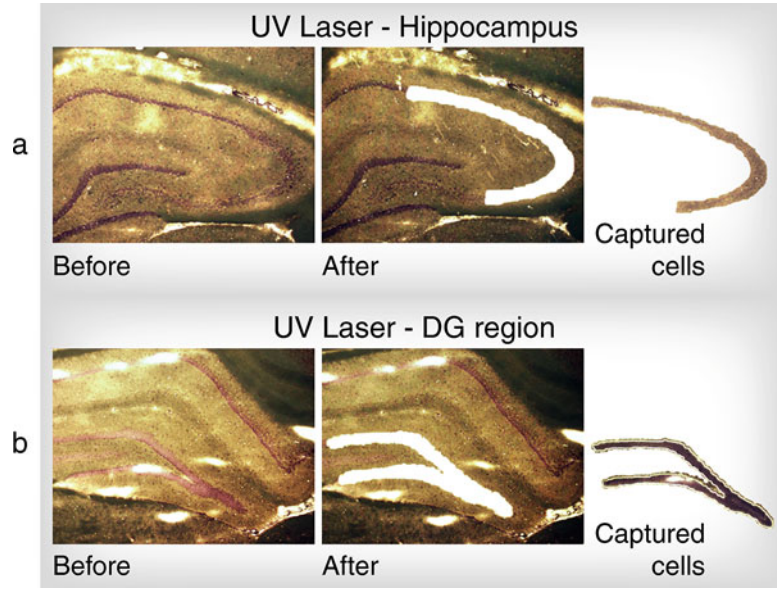

Fig. 1 Laser capture microdissection of hippocampal regions. Before, after, and macro cap images of the (**a**) CA1-CA3 and (**b**) dentate gyrus (DG) subregions of the rat hippocampus. Cells were captured with an IR laser and UV laser from a 30 μM tissue section mounted on a PEN membrane slide

brain regions such as the hippocampal CA1-CA3 subfields and the dentate gyrus (Fig. 1). This system allows us to identify and selectively capture dying (Fluoro-Jade C positive) neurons as well as surviving (Fluoro-Jade C negative) neurons in the hippocampus after experimental TBI. We can use RNA isolated from these laser captured cell samples for a variety of molecular techniques that can be applied to a variety of experiments.

We have successfully completed many studies using these methods. For example, we compared the effects of TBI on the hippocampi of young versus aged rats [4] and mice [5] and found that TBI exacerbated age-related changes in gene expression, suggesting a rationale for how TBI increases the risk for age-related neurodegenerative disorders such as Alzheimer's and Parkinson's diseases [6, 7]. Additionally, following experimental TBI, we found that circadian clock genes were dysregulated in both the hippocampus and suprachiasmatic nucleus [8]. Furthermore, we demonstrated that, in the rat hippocampus after TBI, stochastic gene expression plays a significant role in whether injured neurons (Fig. 2) survive or die [9]. Another application for RNA isolated from laser-captured cell samples is genome-wide transcriptome analysis. We used microarray analysis of laser-captured hippocampal neurons to analyze the effects of neuroprotective compounds as potential treatments for TBI [10] and showed that even with the limited cell samples obtained by LCM we were able to successfully

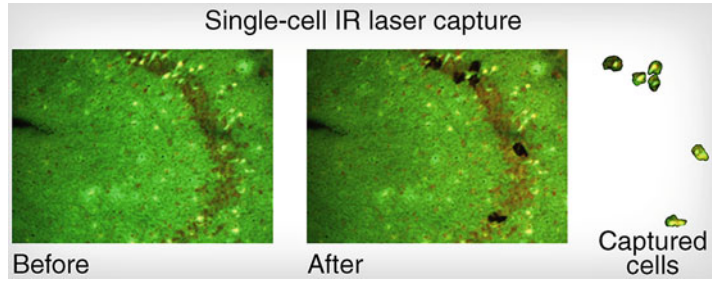

Fig. 2 Laser capture microdissection of single "injured" neurons from the hippocampus. Before, after, and macro cap images of Fluoro-Jade C stained injured neurons. Cells were collected from a 15 μM tissue section mounted on an uncharged glass slide using an IR laser

perform gene expression analysis using pathway-focused PCR arrays [11]. Currently, we are investigating in our TBI model the role that small, noncoding microRNAs play in differentially regulating the expression of their target genes in dying compared to surviving hippocampal neurons (manuscript under review).

The potential for using LCM obtained samples for biological research is unlimited. For example, the ability to profile genomic and proteomic changes in pure populations of cells and in very small sample sizes [12] provides an opportunity to gather tremendous quantities of biologically important data that can be subjected to big data analytical platforms that will be essential for future developments in the diagnosis and management of disease [13].

This chapter describes how we obtain single injured neurons and hippocampal subregions for subsequent downstream genomic analysis. Although the methods described here are specific to the Arcturus^{XT} system, the variety of molecular techniques that can be suitable to the analysis of LCM samples are universally applicable to all LCM samples (provided the samples are properly handled).

2 Materials

2.1 Animals

1. Any experiments involving animals are first approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch, Galveston, Texas in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (8th Edition, National Research Council).
2. Adult male Sprague-Dawley rats, 300–400 g are obtained from vendor Charles Rivers, Portland, Maine.
3. Rats are housed in a vivarium, two per cage and provided food and water ad libitum with these constant conditions: light cycle (600 h–1800 h) temperature (21 °C–23 °C), and humidity (40%–50%) 1 week prior to use.

2.2 Tissue Preparation

1. CM 1850 cryostat (Leica, IL).
2. RNase Zap.
3. Superfrost microscope glass slides, uncharged.
4. Arcturus PEN membrane glass slides (Applied Biosystems, CA).
5. Single-edge razor blades.
6. Disposable Vinyl Specimen Molds 25 mm × 20 mm × 5 mm.
7. Optimum Cutting Temperature "OCT" compound.
8. Disposable, low profile blades.

2.3 Staining of Brain Tissue with Nissl Stain and Fluoro-Jade C

1. Eliminase.
2. RNase Zap.
3. Cresyl Violet.
4. Fluoro-Jade C (Histo-Chem, AR).
5. ETOH/Xylene.
6. Staining dishes and racks.
7. Stericups 0.2 μm filters.

2.4 Laser Capture Microdissection of Single Injured FJ+ Cells and Hippocampal Regions

1. Arcturus^{XT} Laser Capture Microdissection System (Applied Biosystems, CA).
2. Arcturus CapSure Macro Caps (Applied Biosystems, CA).
3. Gene Amp (0.5 ml) thin walled reaction tube (Applied Biosystems, CA).
4. Lysis buffer from RNAqueous Micro Kit (Ambion, TX).

3 Methods

This section will describe the brain tissue preparation for collecting swaths of the hippocampal pyramidal layers and fluorescently labeled single neurons using the Arcturus^{XT} LCM system. Hippocampal pyramidal layers can be captured by using either an infrared (IR) laser or in combination with an ultraviolet (UV) laser for precise region-specific isolation. In contrast, single neurons should preferably be captured using the IR laser as UV rays have the potential to damage the outer cell layer of the tissue where the UV laser cuts and destroys RNA, rendering samples useless for downstream analysis of gene or microRNA analysis. In this protocol, we identify dying neurons in the hippocampus by their affinity for Fluoro-Jade C, a marker of degenerating neurons. Neurons that do not stain with Fluoro-Jade C are presumed to be surviving neurons. This protocol utilizes a method of tissue preparation, staining, and LCM optimized for the RNA analysis of central

nervous system (CNS) tissue which is prone to RNA degradation. It is essential to eliminate any contaminating RNases by thoroughly cleaning the area used for tissue preparation, staining, and LCM. Staining parameters should be amended based on the tissue being used and the specific cell-type sought to be laser captured. It is best to limit the collection of cells using LCM to a specific timeframe of 1 h or less to mitigate the risk of RNA degradation.

3.1 Sectioning of Rat Brain Tissue

1. Remove the brain from the −80 °C freezer and place it into the cryostat at a temperature of −20 °C to allow the tissue to stabilize for approximately 10 min. Orient the brain ventral side up.
2. Slice the brain with a razor blade to remove the posterior portion of the brain just rostral to the cerebellum, and the anterior portion at the optic chiasm. Fill a cryomold with OCT mounting medium, and place the brain into the mold, anterior side down. Allow the brain tissue to freeze in the mounting medium until it turns white.
3. Freeze a specimen disc onto the brain with OCT. Remove the brain from the mold. Insert the specimen disc with brain into the specimen head and tighten the screws.
4. Insert a disposable, low profile blade into the knife holder and tighten the lever down.
5. Move the tissue block closer to the blade and adjust the angle of the tissue block to produce even sections. Tighten the adjustment levers so no tissue block movement occurs during sectioning.
6. Begin slicing the brain to remove the outer layer of OCT and continue until the hippocampal pyramidal layers become apparent. Once the hippocampal region is reached, collect 30 μM (*see* **Note 1**) coronal serial sections on PEN membrane slides; make sure to place the tissue section within the opaque rectangle of the slide. If sectioning for single-cell LCM collect 10–20 μM sections on uncharged microscope glass slides.
7. Preserve the slides at −20 °C in the cryostat until sectioning is complete or the rack is ready for staining (*see* **Note 2**).

3.2 Staining of Brain Tissue for LCM of Hippocampal Pyramidal Layers

1. To remove RNases from glassware, wipe down all staining containers and graduated cylinders with Eliminase and rinse in 18 MΩ water. Prepare all the solutions with RNase-free water and filter the 1% Cresyl Violet stain with a 0.2 μM filter prior to use.
2. Thaw brain sections at room temperature for 30 s and fix the tissue in 75% ETOH for 1 min.

3. For LCM of swaths of hippocampal subregions, after fixation, rinse the slides in RNase-free water (1 min), stain with 1% Cresyl Violet (1 min), rinse in RNase-free water (3 × 1 min), dehydrate with 95% ETOH (2 × 30 s), 100% ETOH (2 × 30 s), and xylene (2 × 3 min).
4. Air-dry all the sections in a fume hood for 15 min prior to LCM. Begin LCM immediately after the sections are dry.

3.3 LCM of Hippocampal Pyramidal Layers

1. Before beginning any LCM experiment be sure to wipe down the area around the instrument and the device itself with an RNase eliminating solution such as RNase Zap to mitigate risk of contamination.
2. Begin by flipping the power switch on the IR Laser base and then flip the power switch for the XT scope.
3. Open and initiate the software on the desktop.
4. Press "Present Stage" in the software's setup panel and the modular stage will move into a position where LCM caps and up to three slides can be loaded. Orient slides with the "frosted" edge to the right to ensure the cap will be offloaded onto the slide in the proper position.
5. Click the "Mem" checkbox in the Cap and Slide handling area next to the respective slide (i.e., A, B, or C) to denote if the slide is a membrane glass slide (*see* **Note 3**).
6. Choose the slide that will be captured first, the software will automatically position itself in the middle of the slide.
7. From the "Toolbar" select "Image," then select "Acquire Overview." The camera will then take a tiled image which can be used for tissue location and orientation for cap placements (*see* **Note 4**).
8. With the cursor, click on the area in the tiled image where LCM will occur, right click on the spot, and select "Place Cap at Region Center." The software will then automatically place the cap over the selected position.
9. In order to ensure proper alignment of the IR laser the IR spot must first be located on a blank portion of the slide. Place the blue cross over a blank area.
10. Click the "i" in the "Microdissect Tools Pane" to open a systems dialog box, then click the "IR Locate tab" (*see* **Note 5**).
11. Fire an "IR Test Shot" to gauge the size of the spot, right click with the cursor in the middle of the spot, and then select "Locate IR Spot."
12. The spot size can then be adjusted to match the size of the individual cell or other tissue types by manually altering the

"Power, Diameter, or Duration" on any of the spot sizes or by moving the sliding scale at the bottom of the dialog box (*see* **Note 6**).

13. If using the UV laser on membrane slides, at this point choose the "UV Locate" tab in the open systems dialog box. When the UV spot is visible click on the spot and the UV laser is located. The UV laser is in a fixed position with the IR so any time the user would like to move to another part of the tissue there is no need to re-locate the UV.
14. If the user intends to capture an entire region (e.g., hippocampal pyramidal layers) click the "Freehand Drawing option" (square with a pen) in the "Select Tools Pane."
15. Carefully draw around the perimeter of the region making sure not to lose contact between the stylus and the screen and also that the beginning and end-point connect.
16. The software will automatically lay down IR spots within the UV cutting range; but, the user may choose to lay down other spots to ensure that the tissue is adhered to the cap after UV cutting, selecting the "Single IR Spot", or "IR Spot Line" from the "Select Tools Pane."
17. Before UV cutting the user can adjust the speed at which the UV laser cuts by moving the sliding scale located in the "Microdissect Tools Pane" (*see* **Note 7**).
18. When the UV cutting area and the IR spots are in place, press the Cut and Capture button in the "Microdissect Tools Pane." The user can choose whether to have IR spots fired first or UV laser cutting completed first in the "Systems Dialog box" mentioned earlier.
19. Once the UV laser and the IR laser have cut and fired, respectively, press the "Move Cap to QC" button located in the "Microdissect Tools Pane" in order to view the cut tissue. Alternatively, this step can be omitted if the user has more slides to collect in which case **steps 6–20** should be repeated.
20. When finished with collection make sure the cap is in the QC position and click "Present Stage." When the cap is removed from the QC position make sure to unselect that cap position from the QC position so that the software does not continue to recognize a cap in that position.
21. Immediately place LCM Macro Cap with adhered tissue into the relevant lysis buffer and store at −80 °C until used for RNA isolation and downstream analysis.

3.4 Staining of Brain Tissue for Laser Capture Microdissection of Single FJ+ Neurons in the Hippocampus

1. Prepare all solutions RNase-free as described in Subheading 3.2.
2. For LCM of single injured neurons fix tissue sections in 75% ETOH (1 min), rinse the slides in RNase-free water (1 min), counterstain with 1% Cresyl violet (15–20 s), rinse in RNase-free water (2 × 30 s), stain with Fluoro-Jade C (4 min), rinse in RNase-free water (3 × 1 min), dehydrate with 95% ETOH (30 s), 100% ETOH (30 s), and xylene (2 × 3 min).
3. Air dry sections in a fume hood for 15 min prior to LCM. Begin LCM immediately after sections are dry.

3.5 Single-Cell LCM of Fluorescently Labeled Dying Versus Surviving Hippocampal Neurons

1. Before moving forward with single-cell capture, turn on the fluorescence module and move the associated filters (located in the filter turret) into place.
2. Press the "Fluorescence icon" located in the "Inspect Tool Pane." A pop-up window will appear asking if you would like the Arcturus^{XT} instrument to take control of the illumination source. If the XT device has a kick pedal, press "No", and depress the pedal to turn the fluorescence on otherwise, select "Yes". Fluorescence settings such as lamp intensity, camera gain, and exposure time can be manipulated from the "Inspect Options" dialog box after selecting the Fluorescence tab.
3. Be sure to locate the IR laser, as described earlier, before capturing individual cells.
4. The Arcturus^{XT} software allows cells or tissue to be selected for LCM from a static image so that fluorescence can be turned off to avoid photo-bleaching. After adjusting the slide image into focus, select "Timed Exposure" from the "Inspect Options dialog box." The shutter will only remain open long enough to capture an image for LCM and will then close to avoid photo-bleaching. Alternatively, you can bypass pressing "Timed Exposure" by simply clicking "Camera" to capture a static image and then manually close the shutter.
5. Capture Fluoro-Jade positive (FJ+) labeled cells and the same number of Fluoro-Jade negative (FJ-) cells on separate LCM Macro Caps (absolute numbers will depend on your downstream applications).
6. Immediately place captured cells in the correct lysis buffer for your RNA isolation procedure and store at −80 °C until ready for isolation and downstream analysis (*see* **Note 8**).

4 Notes

1. When using the UV laser on Pen Membrane slides it is possible to cut upward of 100 μM thick sections. We find that the optimal thickness is around 30 μM for UV cutting of brain

tissue sections because slicing thicker sections on the cryostat requires higher temperatures. Raising the ambient cryostat temperature to −15 °C or greater is not recommended due to possible RNA degradation.

2. When performing LCM on brain regions, because of the time it takes to cut, stain, and manipulate the software, we advise not cutting an entire rack of sections. We have found that anywhere from 6 to 10 sections per cap is a reasonable amount to collect. However, as you become more comfortable with the software and quicker with its operation, collecting from more sections may be possible.
3. The UV laser cutter will not operate on a non-membrane slide.
4. It is not entirely necessary to perform an "Image Overview". For quick procedures this step can be omitted and specific locations can be determined by the operator and scrolled to manually.
5. The IR spot can also be located on the primary screen by placing the blue cross on a non-tissue area and using the "IR Capture Test Spot" icon in the "Microdissect Tools Pane." Once the test spot has been fired and the size adjusted, as mentioned in the procedures, place the cursor in the middle of the spot, right-click, and select "Located IR Spot."
6. It may be necessary to fire several test spots in order to ensure proper spot size after adjusting the power and duration in the "IR Spot Size" tab from the "Microdissect Options" dialog box. We advise starting with the smallest spot size and then adjusting up as necessary. This is especially important when collecting single cells to minimize the risk of contaminating with non-target cells.
7. Cutting at slower speeds will produce a wider UV band so take into consideration if the tissue to be collected has the potential to be damaged, as UV will destroy RNA in cells it cuts through. It may be necessary to cut with the UV laser two or three times to ensure that the membrane detaches from the slide. Cutting at a faster speed will allow this to be done more quickly.
8. For molecular analysis of mRNA from single-cell populations, it may be necessary to perform a pre-amplification step before gene expression analysis. We use the RT^2 Nano Pre Amp cDNA synthesis kit (Qiagen) to pre-amplify mRNA targets from as little as 1 ng of total RNA. To perform analysis of microRNA alterations in single cells using pathway-focused PCR arrays, we use the miScript Single Cell Kit (Qiagen) to measure microRNA expression from as few as 10 cells. As most microRNA qPCR arrays require at least 1–10 ng of RNA for a reverse transcription reaction, LCM of a limited number of cells may not provide adequate RNA and must therefore be

pre-amplified. The miScript Single Cell kit protocol can also be amended for reverse transcription and pre-amplification of microRNA from LCM swaths. We advise the use of quality control PCRs for protocol steps provided by the manufacturer to ensure efficiency of reverse transcription and pre-amplification.

9. Although several LCM systems exist, their common purpose is to rapidly obtain identifiable cell types or cell populations of interest that can be further analyzed using modern molecular analysis methods such as qPCR, microarray, digital PCR, RNA-seq, and mass spectrometry. It is important to recognize that the sample preparation and laser capturing techniques need to be optimized for each tissue and cell type. The methods presented here are specific for rat brain tissue and have worked well for our brain injury studies over the past 15 years.
10. One specific application of using LCM for obtaining RNA from different brain regions is in the area of biomarker discovery. We are comparing gene expression in different brain areas obtained by LCM with expression of the same genomic markers in biofluids in TBI animals with the goal of defining circulating biomarkers of regional brain damage.
11. We have used LCM combined with single-cell amplification techniques to study TBI comorbidities. Our studies have shown that specific genes associated with comorbid diseases such as depression, i.e., SLC6A15 [14], are highly expressed in dying hippocampal neurons after TBI [9]. We have studied gene and miRNA expression in laser-captured neurons from four distinct brain regions linked to depression- the rat hippocampus, prefrontal cortex, nucleus accumbens, and suprachiasmatic nucleus- and found that several TBI-dysregulated miRNAs that are differentially expressed in these brain regions have previously been linked to the pathophysiology of depression [15].

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Chapter 14

Isolation of Distinct Types of Neurons from Fresh Brain Tissue Using Laser Microdissection in Combination with High-Performance Liquid Chromatography—Mass Spectrometry

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Abstract

Humans age and the ageing process affects cells in all areas of the human body, including nerve cells within the brain. With advancing age there is also a rise in the probability of developing a neurodegenerative disorder such as, e.g., amyotrophic lateral sclerosis, Huntington's disease, Parkinson's disease, or Alzheimer's disease. In all these age-related neurodegenerative disorders, distinct neuron populations within specific brain regions are primarily affected. For example, Parkinson's disease is characterized by a slowly progressive degeneration of dopaminergic neurons in the *substantia nigra* whereas the *entorhinal cortex* is first affected in Alzheimer's disease. In patients suffering from Huntington's disease, neurons in both *striatum* and *cortex* undergo substantial cell loss and in amyotrophic lateral sclerosis the neurodegeneration arises from the spinal cord and the motor cortex. For the investigation of the differences in neuronal vulnerability, it is important to examine the protein expression pattern in these specific neural populations. By this, conclusions about the origination process of these diseases can be achieved. In order to obtain this objective, specific isolation of distinct neurons from the surrounding brain tissue is indispensable. However, discrimination as well as isolation of distinct types of neurons can be challenging, due to the brain tissue's complexity. With traditional methods such as the homogenization of tissue samples, a specific isolation of single neuron populations is not feasible because homogenization results into a mixture containing all cell types. Laser microdissection can overcome this technical limitation. First, this method enables visualization of tissues via a microscopic unit and therefore an enhanced discrimination of different brain cells. Second, a laser device guarantees a contact-free and consequently a contamination-free separation of distinct neurons from the surrounding brain tissue. In the following, we present a detailed protocol that includes a workflow for the isolation and analysis of neurons from freshly frozen post mortem human brain tissue samples. During this procedure, the brain tissue is sectioned, stained, laser microdissected, and ultimately analyzed by high-performance liquid chromatography—mass spectrometry.

Key words Laser microdissection, Proteomics, Mass spectrometry, Frozen fresh tissue, Neurons, Neurodegeneration, Neurodegenerative diseases

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1 Introduction

Ageing is defined as a natural, non-reversible ongoing process causing various changes in an organism. These changes are mostly associated with the loss of function such as cellular senescence or degeneration [1–5]. Due to the changes in the physiology of the body ageing is the major risk factor for the development of various diseases [6]. Especially, neurodegenerative diseases such as the sporadic forms of Parkinson's disease and Alzheimer's disease are correlated with ageing. These disorders mainly occur in elderly above the age of 50 years [2] whereby the probability to suffer from Alzheimer's disease is highest from the age of 85 years and above the age of 70 years for Parkinson's disease [1]. In addition, there is also a significant rise of the probability to suffer from amyotrophic lateral sclerosis (ALS, also known as Lou Gehrig's disease) above the age of 40 years [1]. The cause of these neurodegenerative disorders still remains unclear but the major underlying reason for the development seems to be a complex interplay of genetic and environmental factors [7]. These factors affect several essential cellular pathways resulting in the loss of distinct neuron populations, which is a characteristic for all neurodegenerative disorders. Moreover, neurons in a specific region are primarily affected in each disease. For example, in Parkinson's disease initially the dopaminergic neurons of the *substantia nigra* undergo substantial cell loss [2] before the neurodegeneration also spreads to other regions in later stages of the disease [1]. As a result, neurodegenerative alterations can also be found in wide parts of the central as well as enteric nervous system. A closer look at the primarily affected neuronal populations in patients suffering from the respective neurodegenerative disorder might explain why the neurodegeneration originates from this particular area. Therefore, it is necessary to isolate these specific neurons from the brain. By isolation of defined neurons from brain tissue, it is possible to study their function and influence on the entire system. However, human brains have a very complex structure and can be divided into various regions including over 100 billion highly specialized and interconnected cells [4, 8]. Thus, the isolation of a specific cell population can be an extremely challenging task. For example, in the past, it was not possible to separate specific cell types out of a complex tissue because many isolation approaches are based on the homogenization of cell tissues. Homogenization leads to a mixture that contains all cell types [9] and is usually based on mechanical or chemical methods with harsh conditions often resulting in damaged cells. As a consequence, investigation of a specific cell population is not possible. Due to the mixture's heterogeneity it is not feasible to specifically separate intact cells without a large amount of contamination such as other neural species. These contaminations of, e.g.,

undesired neural cells could overlay signals of minor cell populations in mass spectrometric studies, thereby masking wanted hints. The invention of laser microdissection helps to overcome these technical limitations [4]. The technique allows a contact-free isolation of not only distinct neuron types but also of any other specific cell type, single cells, or cell compartments from the surrounding tissue. Further, advantages of laser microdissection are the precision [10, 11] and the variety of application areas [4]. In addition, due to the minor willingness for organ donation it is beneficial that only a small amount of tissue samples is required for the performance of a conclusive mass spectrometric analysis. This means that for a single-neuron analysis with significant results at least 100 ng of protein must be employed from the tissue. This can be achieved by isolation of ~2500 neurons (1,500,000 μm [2]) from 10 μm thick sections. For the analysis of a complete brain area, e.g., the *substantia nigra*, 25,000,000 μm [2] of *substantia nigra* tissue from 20 μm thick sections are sufficient [4]. The isolation via laser microdissection is performed by directly studying the tissue sample through light microscopy. This microscopic visualization enables an uncomplicated classification, differentiation, and marking process of distinct neurons. Via live observation of the tissue during the laser and collection step, the method can be adjusted directly to the respective tissue sample. In this way, the precise isolation of specific elements, e.g., different types of neurons is guaranteed. By proteomic studies using mass spectrometry, the proteome of the isolated neurons can be investigated. These studies enable conclusions about the protein function and their effect on various pathways. Thus, questions such as "Why do only some people suffer from neurodegenerative diseases when growing older while others age healthily?", "Why are the dopaminergic neurons of the *substantia nigra* primarily affected during the neurodegeneration process in Parkinson's disease?" and "What exactly causes the selective neuronal vulnerability for each disease?" might be answered.

2 Materials

2.1 Tissue Sectioning

1. Cryostat.
2. Stainless steel knife.
3. Tissue holder.
4. 1.0 Poly-ethylene-naphthalene (PEN) membrane slides (Carl Zeiss Microscopy GmbH, Göttingen, Germany).
5. Storing boxes for membrane slides.

2.2 Cresyl Violet Staining

1. 200 mL Schott flask.
2. Magnetic stirring plate.

3. Agitator.
4. Sterile filtering device.
5. 50 mL conical tubes.
6. Stand for 50 mL conical tubes.
7. Pipette and pipette tips (20–200 μL).
8. Richard-Allan Scientific™ Neg.50™ Frozen Section Medium.
9. Acetone.
10. Cresyl violet acetate.
11. 50% (v/v) ethanol.
12. 70% (v/v) ethanol.
13. 100% (v/v) ethanol.

2.3 Laser Microdissection and Pressure Catapulting

1. PALM MicroBeam—System (Carl Zeiss Microscopy GmbH, Oberkochen, Germany) (*see* **Note 1**).
2. Sample collection caps (MicroTube500) (Carl Zeiss Microscopy GmbH, Oberkochen, Germany) (*see* **Note 2**).
3. Computer with PALM MicroBeam corresponding software (PALMRobo 4.6 pro).
4. Touch pad with touch pen.
5. Pipettes and pipette tips (2–20 μL, 20–200 μL) (*see* **Note 3**).

2.4 Tryptic Digestion

1. Centrifuge 5415R with fixed-angle rotor (Eppendorf AG, Hamburg, Germany).
2. Thermomixer (Eppendorf AG, Hamburg, Germany).
3. Inert mass spectrometric glass vial inlets.
4. Vacuum concentrator RVC2-25 CD plus (Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany).
5. Cryobox.

2.5 High-Performance Liquid Chromatography—Mass Spectrometry

1. High-performance liquid chromatography system (e.g., the UltiMate 3000 RSLC nano LC system (Dionex, Idstein, Germany)).
2. Mass spectrometer (e.g., Q Exactive system (Thermo Fisher Scientific, Bremen, Germany)).
3. Xcalibur Software (Thermo Fisher Scientific, Bremen, Germany).
4. Inert mass spectrometric glass vial.
5. Mass spectrometric data analysis software for proteomics (e.g., Proteome Discoverer (Thermo Fisher Scientific, Bremen, Germany) or MaxQuant).
6. 0.1% Trifluoroacetic acid.
7. 84% Acetonitrile.

2.6 Tryptic Digestion

1. RapiGest™ 1% (Waters, Milford, USA).
2. 250 mM 1,4-dithiothreitol (AppliChem GmbH, Darmstadt, Germany).
3. 0.55 M Iodoacetamide.
4. Trypsin (Promega, Mannheim, Germany).
5. 10% Trifluoroacetic acid.
6. 0.1% Trifluoroacetic acid.

3 Methods

3.1 Tissue Sectioning

1. Precool the cryostat to an object temperature between −10 °C and −15 °C and a cryostat chamber temperature of −20 °C (Fig. 1).
2. Install the stainless steel knife with an angle of 9° (*see* **Note 4**).
3. Clean the stainless steel knife with acetone.
4. Place a storing box for membrane slides in one corner of the cryostat.
5. Transfer the human brain tissue from the −80 °C freezer to the cryostat using an icebox.
6. Let the human brain tissue adjust to the cryostat chamber temperature for 15 min.
7. Label membrane slides with an unambiguous assignment using a pencil (*see* **Notes 5** and **6**).
8. Place a small drop of the frozen section medium on the tissue holder and let it freeze on (*see* **Note 7**).
9. Set the human brain tissue onto the frozen section medium on the tissue holder, when it is almost completely frozen and let the frozen section medium harden (*see* **Note 8**).
10. Install tissue holder onto the cryostat and orientate the holder for optimal sectioning.
11. Adjust cutting settings of the cryostat to a section thickness of 20 µm.
12. Trim human brain tissue until the desired section plane is reached.
13. Adjust cutting setting of the cryostat to a section thickness of 10 µm (*see* **Note 9**).
14. Cut two sections and discard them (*see* **Note 10**).
15. Cut a section of the tissue.
16. Place the tissue section carefully on the membrane slide (*see* **Notes 11** and **12**).

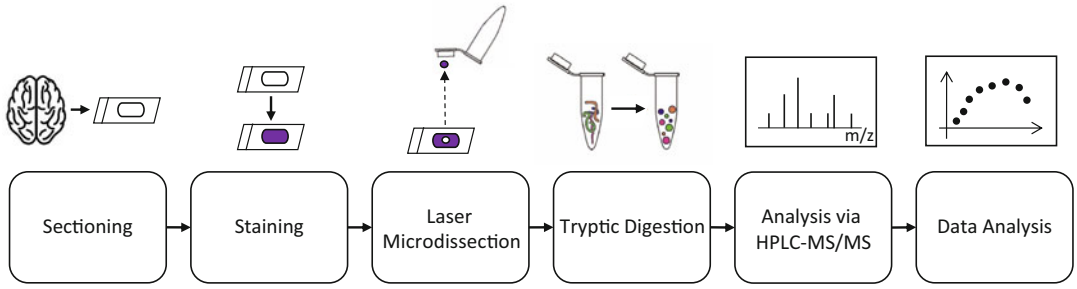

Fig. 1 Workflow. The isolation and analysis of distinct types of neurons from freshly frozen brain tissue is performed via six steps. First, the frozen tissue is cut into 5–20 μm sections via a cryostat. Before laser microdissection (LMD) is performed, the sections are staining using a cresyl violet staining solution. Via LMD the neurons are isolated from the surrounding brain tissue. The collected cells are processed via tryptic digestion and ultimately the proteomic analysis is performed via high-performance liquid chromatography in combination with tandem mass spectrometry. For the final data analysis different software programs can be used

17. Store tissue slides placed on membrane slides in storing box for membrane slides until sectioning is completed.
18. Transfer the storing box for membrane slides into a −80 °C freezer using an ice box.

3.2 Cresyl Violet Staining

1. Place a 200 mL Schott flask onto the fine scale and reset it.
2. Weigh 1 g cresyl violet acetate into the 200 mL Schott flask.
3. Dissolve cresyl violet acetate in 100 mL 50% (v/v) ethanol.
4. Stir the solution overnight on a magnetic stirring plate using an agitator.
5. Filter the cresyl violet staining solution by sterile filter device to remove undissolved particles.
6. Fill two 50 mL conical tubes with 70% (v/v) ethanol (precooled to 4 °C) and one 50 mL conical tube with 100% (v/v) ethanol (precooled to 4 °C) and place them into a stand for 50 mL conical tubes (*see* **Note 13**).
7. Transfer tissue slide from −80 °C freezer into the first 50 mL conical tube filled with 70% (v/v) ethanol.
8. Incubate for 2 min.
9. Place the tissue slide onto a tissue wiper and let it dry for a few seconds.
10. Cover tissue section with 200 μL cresyl violet staining solution.
11. Incubate for 20 s.
12. Discard cresyl violet staining solution.
13. Wash tissue slide by dipping three times into the second 50 mL conical tube filled with 70% (v/v) ethanol.

14. Dip tissue slide once into 50 mL conical tube filled with 100% (v/v) ethanol.
15. Dry the tissue slide on a tissue wiper.
16. Stained tissue sections are directly used for laser microdissection and pressure catapulting.

3.3 Laser Microdissection and Pressure Catapulting

1. Switch on the PALM MicroBeam—System (*see* **Note 14**).
2. Insert stained tissue section into the holder of the RoboStage II.
3. Adjust the magnification of the microscope to 50×.
4. Carry out an overview scan of the tissue section by using the scan function in the navigator window of the software interface (*see* **Note 15**).
5. Highlight the area of interest manually by using the laser setting "Cut" (*see* **Note 16**).
6. Adjust the magnification of the microscope to 400×.
7. Mark cells of interest manually using the laser setting "RoboLPC" (*see* **Notes 17–22**).
8. After the marking is completed control whether markings are still in place, adjust if necessary manually and save the markings.
9. Adjust laser settings using an area of the tissue that is not needed for the analysis (*see* **Notes 23** and **24**).
10. Fill sample collection cap with 47 µL ultrapure water (*see* **Notes 25–28**).
11. Insert the sample collection cap into the collector of the RoboMover.
12. Position the RoboMover above the RoboStage II using the software's interface (*see* **Note 29**).
13. Start the laser (*see* **Note 30**).
14. Lower light intensity of the microscope to reduce evaporation of ultrapure water (*see* **Note 31**).
15. Control energy and focus settings during the laser process and adjust properties if necessary (*see* **Note 32**).
16. When sampling is completed navigate the RoboMover to its starting position (*see* **Note 33**).
17. Remove the sample collection cap carefully (Fig. 2).
18. Close the sample collection cap carefully.
19. Store sample collection cap upside down in a cryobox that is placed into a −80 °C freezer.

3.4 Tryptic Digestion

1. Precool centrifuge to 4 °C.
2. Transfer samples from −80 °C freezer into the precooled centrifuge.

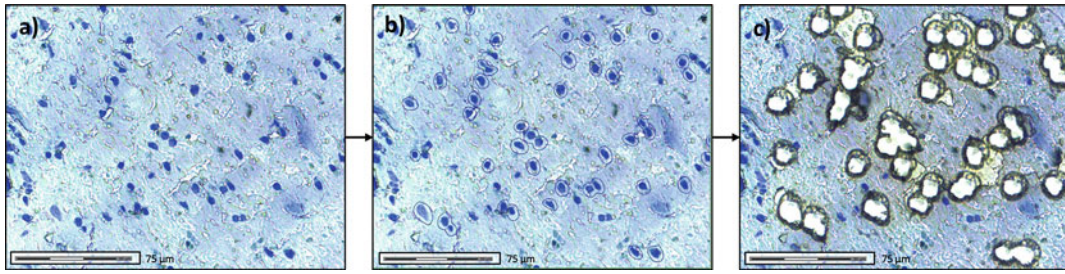

Fig. 2 Laser microdissection of *substantia nigra* tissue. Neurons are marked manually in 400× magnification using the touch pad and the touch pen of the PALM MicroBeam system. After complete marking, the neurons are separated from the surrounding tissue using a laser beam followed by collection in the overhanging collection cap via a defined laser pulse. The images show the tissue before laser microdissection (**a**), with markings (**b**) and after laser microdissection (**c**) in 400× magnification

3. Centrifugate samples at 2655 × *g* at 4 °C for 1 min to transfer the sample from the cap of the sample collection cap to its body.
4. Add 7 µL of 1% RapiGest™ and incubate for 30 min at room temperature.
5. Preheat the thermomixer to 60 °C.
6. Incubate the samples for 30 min in a thermomixer at 60 °C and 300 rpm.
7. Add 1 µL of 250 mM 1,4-dithiothreitol to the samples and incubate for 30 min in a thermomixer at 60 °C and 300 rpm.
8. Centrifugate the samples shortly to ensure that there is no liquid left in the cap due to condensation during the heating steps.
9. Let the samples cool down to room temperature for 15 min.
10. Add 1.4 µL of 0.55 M iodoacetamide to the samples and incubate for 30 min in the dark at room temperature.
11. Preheat the thermomixer to 95 °C.
12. Incubate the samples for 5 min in a thermomixer at 95 °C and 300 rpm.
13. Centrifugate the samples shortly to ensure that there is no liquid left in the cap due to condensation during the heating steps.
14. Let the samples cool down to room temperature for 15 min.
15. Preheat the thermomixer to 37 °C.
16. Add 1:4 trypsin to the samples (*see* **Note 34**).
17. Incubate the samples for 4 h in a thermomixer at 37 °C and 300 rpm (*see* **Note 34**).
18. Add 3.25 µL of 10% trifluoroacetic acid to the samples (*see* **Note 35**).

19. Incubate the samples for 30 min in a thermomixer at 37 °C and 300 rpm.
20. Precool centrifuge to 4 °C.
21. Centrifugate the samples at 10621 × *g* at 4 °C for 15 min to remove unwanted cell components (*see* **Note 36**).
22. Transfer the supernatant of samples into inert mass spectrometric glass vial inlets.
23. Narrow samples down to 25 μL using a vacuum concentrator (*see* **Note 37**).
24. Fill the samples up to a final volume of 35 μL with 0.1% trifluoroacetic acid (*see* **Note 38**).
25. Place the samples into a cryobox and store them in a −80 °C freezer until further usage.

3.5 High-Performance Liquid Chromatography and Mass Spectrometry

1. The high-performance liquid chromatography system (e.g., the UltiMate 3000 RSLC nano LC system from Dionex, Idstein, Germany) is coupled online to the mass spectrometer (e.g., Q Exactive system from Thermo Fisher Scientific, Bremen, Germany), so that the samples are transferred automatically.
2. Adjust following settings for the high-performance liquid chromatography system by using the software Xcalibur: Trap column (*see* **Note 39**). Temperature: 60 °C., Flow rate: 30 μL/min. Running buffer: 0.1% trifluoroacetic acid. Analytical C18 reversed-phase column (*see* **Note 40**). Temperature: 60 C.Flow rate: 30 μL/min. Running buffer A: 0.1% trifluoroacetic acid. Running buffer B: 84% acetonitrile. Gradient: 5–40% running buffer B over 98 min.
3. Adjust the following settings for the mass spectrometer by using the software Xcalibur: General settings.Scan range: 350–1400 m/z. Resolution: 60,000 (at x m/z). Internal recalibration. Target AGC of 3e6. Fill time: 80 ms. Look mass: Polydimethylcyclosiloxane (m/z of 455.12002). MS/MS analysis of m/z values. Dynamic exclusion list: 30 s, top 10 ions (charge: +2, +3, +4); Fragment ion analysis. Resolution: 30,000 (at x m/z); Target AGC of 1e6 Fill time: 120 ms.
4. Prepare the samples by dissolving 200 ng of sample peptides in a volume of 15 μL 0.1% TFA in inert mass spectrometric glass vial inlets (*see* **Note 41**).
5. Place inert mass spectrometric glass vials inlets into inert mass spectrometric glass vials.
6. Place the samples on the autosampler of the high-performance liquid chromatography system (*see* **Note 42**).

7. The high-performance liquid chromatography and mass spectrometry measurement is performed automated. If using the same system as indicated proteomic analysis is possible (*see* **Note 43**).
8. Analyze achieved RAW data using a proteomic suitable software, where achieved RAW data are compared to a proteomic reference data bank (e.g., Uniprot) via an algorithm (e.g., Mascot) (*see* **Note 44**).
9. Following statistical data analysis is carried out regarding the respective question (*see* **Note 45**).

4 Notes

1. The PALM MicroBeam—System should contain following equipment: RoboMover with a collector (e.g., Single Tube Collector 500 μL), RoboStage II with a holder (e.g., Three Slide Holder). An incubation chamber prevents contaminations from the environment (e.g., dust or other particles contained in the air). Further, the corresponding software (PALMRobo 4.6 pro) has to be installed.
2. Different collection devices are available. Not only different sizes of MicroTubes (200 μL, 500 μL), but also with or without adhesive material in the cap (could lead to interferences during mass spectrometry) are available as well as petri dishes for living cell experiments. Consider that the collector has to be suitable for the collection device.
3. Use pipette tips with extended tips. By those filling of the sample collection cap is easy to handle.
4. Handle the stainless steel knife carefully due to danger of injury. Never touch the sharp site of the stainless steel knife.
5. Handle membrane slides with care. If the membrane gets damaged it cannot be used for laser microdissection, since it would absorb moisture (e.g., staining solution or water).
6. The assignment could contain the case number, the section number, section thickness, and the date of sectioning. Using a pencil will prevent blurring of the assignment during tissue staining steps.
7. Frozen section medium should not be completely harden.
8. Ensure that no frozen section medium contacts parts of the tissue which should be used for analysis, since frozen section medium disturbs mass spectrometric analysis.
9. Clean the knife in between by using brushes or if heavy contaminations of the knife are present with acetone.

10. It cannot be guaranteed that first tissue sections after a change of section thickness have the exact section thickness.
11. Place just one section on each slide.
12. Narrow the membrane slide slowly toward the tissue section until it attaches to the membrane slide. Avoid folding of the tissue.
13. Change ethanol solutions regularly, at least after each case.
14. Laser microdissection should be performed in a temperature-controlled environment to ensure optimal and reproducible conditions.
15. Adjust scan area to the size of section to eliminate unneeded areas of the slide. Set the auto focus for every three pictures to guarantee a precise image. Consider that on the one hand the light intensity should not be too high to prevent the tissue from heating up and on the other hand that the overview scan takes longer if the exposure time is too high. A good compromise for overview scan settings is a light intensity of 25–30 and an exposure time of 11 ms.
16. Set the elements of this marking to skip.
17. Process the area of interest schematically, so that no part is missed.
18. Using a touch pad as well as a touch pen simplifies the accurate marking of cells.
19. Use different colors for different elements.
20. If differentiation of cells is difficult it might help to change the focus or the light intensity.
21. Mark elements of interest by drawing around their contour. There should be a small space between the line and the element regarding the width of the laser beam to ensure that the element of interest is separated from the tissue without causing damages on the cell.
22. Save markings during process.
23. Tissue slides can be stored at −80 °C in urgent cases (e.g., overnight), but not if the laser process was partially performed.
24. Before catapulting the laser should cut out the elements precisely. Following settings have proven their value for 10 μm thick tissue sections: Cut Energy 36 ± 5, Cut Focus 56 ± 5, LPC Energy 22 ± 1, LPC Focus −4 ± 1. Use an area of the tissue that is not needed for analysis. Additionally, if needed the software PALMRobo 4.6 pro provides a half-automated calibration of laser settings.
25. Control that the sample collection cap is completely filled with ultrapure water.

26. When handling the sample collection cap wear gloves that are one size smaller than your normal size to prevent contact of folds of the gloves to the water.
27. Handle the filled sample collection cap carefully; otherwise, ultrapure water can drop out.
28. Hold the sample collection cap upside down to ensure that no contaminations fall into ultrapure water.
29. Ensure a sufficient distance between the sample collection cap and the tissue slide. Otherwise, ultrapure water could touch the tissue and would drop out of the sample collection cap.
30. For greater elements the Center RoboLPC option of the laser is recommended.
31. Make sure that there is always enough ultrapure water in the sample collection cap to ensure that there is no loss of sample material. Refill if necessary. Therefore, navigate the RoboMover to its starting position, remove the sample collection cap carefully. Add a small drop (approx. 5 μL) of ultrapure water to the filled cap without touching it with the pipette tip.
32. Manual LPC is possible, if an element is not separated from the tissue automatically via the laser pulse.
33. Collect elements until having a total amount of at least 1 million μm [2] (better 1.5 million μm [2]) of neurons to ensure there is enough material for measurements. The total amount of the collected elements per slide can be observed in the "summary" section of the element list of the software program. The total collection time per section including marking of the elements and the following laser step is 6 h on average but highly depends on the section.
34. Depending on your samples the amount of trypsin and the time of digestion can variate. Determine optimal digestion conditions for your studies via pre-studies.
35. Trifluoroacetic acid leads to altered pH conditions, which inactivates trypsin.
36. Fragments of the membrane of the membrane slides are cut out and collected during laser microdissection. These have to be removed, since they can lead to disturbances during the high-performance liquid chromatography—mass spectrometry measurement.
37. Complete drying of the samples could lead to a loss of peptide amount, because it is difficult to dissolve peptides back into the solution.
38. Samples should be diluted in a solvent that is compatible with the used high-performance liquid chromatography—mass spectrometry setup.

39. A C18 100 μm × 2 cm trap column with a particle size of 5 μm and a pore size of 100 Å is recommended in this protocol.
40. A C18 75 μm × 50 cm analytical column with a particle size of 2 μm and a pore size of 100 Å is recommended in this protocol.
41. Before proceeding high-performance liquid chromatography and mass spectrometry use a method of choice for peptide concentration analysis to ensure that the correct amount of sample is transferred to the system (e.g., by amino acid analysis).
42. The autosampler cools samples at 4 °C. When analyzing a big cohort of samples place those by and by.
43. Settings highly depend on the system used for the analysis and the analyte. It has to be adjusted individually for each type of sample. The total measuring time for each sample according to the described protocol is 2 h followed by a 1 h washing step.
44. Different software for proteomic analysis of mass spectrometry is available, for example Proteome Discoverer 1.4 or MaxQuant, which use different algorithms (Mascot and Andromeda, respectively).
45. For the statistical data analysis following parameters should be considered: the false discovery rate should be smaller than 0.1%, the analysis of variance (calculated using a *t*-test or ANOVA test) should have a p-value smaller than 0.05% and the fold change should be twice the technical variance to ensure biological variance.

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Chapter 15

Immuno-Guided Laser-Capture Microdissection of Glial Cells for mRNA Analysis

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Abstract

Laser-capture microdissection (LCM) allows for retrieval of specific cell populations in situ. By combining immunofluorescent labeling with LCM, mRNAs can be probed by qRT-PCR for determining in situ gene expression during health and disease. This approach permits obtaining and analyzing histologically enriched cell populations in a tissue that can be hardly obtained from other methods such as white matter astrocytes from rodents or any individual cell population from archival human or rodent brain tissues. Herein, we present our methodology of laser-captured mouse spinal cord white matter astrocytes, which can be adapted for any cell type in CNS tissue and low RNAse containing tissues. The methods presented with an emphasis on tips and advices include the cryostat section preparation from snap-frozen tissue, an adapted immunofluorescent labeling, a brief overview of LCM using a UV-based technology with polyethylene membrane glass slides, procedures for direct use of RNA from lysis buffer vs. column-based purified RNA, RNA quality/quantity assessment, the reverse transcription and preamplification steps used before real-time qPCR analysis.

Key words Astrocyte, Real-time PCR, RNA integrity, Gene expression, Transcriptome, Laser-assisted microdissection (LMD)

1 Introduction

Probing transcripts from selected cell populations by immune-guided laser-capture microdissection (LCM) coupled to quantitative RT-PCR is an approach that can give important in situ gene expression information during health and disease [1–3]. Though the technique can be time consuming, it offers an alternative to RNA analysis to Fluorescence-Activated Cell Sorting (FACS)-enriched specific cell populations, especially when adequate FACS markers or fresh tissues are not available. Indeed, rodent white matter astrocytes (especially resting astrocytes entrapped in myelin) cannot be well purified by FACS methods, in contrast to cortical gray matter astrocytes [4]. However, when using conventional

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techniques for working with trace amounts of nucleic acids, loss of product during purification steps is a frequent problem. Then, the number of microdissected cells required for analysis renders the procedure long and tedious. To reduce this loss, the addition of RNA carrier—usually polyA—into the sample is recommended [5]. However, the polyA RNA carrier is recovered with the mRNA extract from the Qiagen microkit or Macherey-Nagel RNA XS columns and such a carrier is incompatible with several downstream applications such as Takara pre-amplification that we use. Despite this high RNA loss when using minute amount of starting material, the procedure yields however pure and intact mRNA that can be analyzed directly by 2100 Bioanalyzer Nanodrop (to accurately assess RNA concentration if it is superior to 2 ng/μl) or via an Agilent RNA 6000 Pico LabChip kit for quality control of total RNA in a range from 200 to 5000 pg/μl (though lower concentration down to 50 pg/μl may also allow sufficient appreciation of RNA quality). By pooling several sample extracts, we reach sufficient total RNA (5 ng) for qRT-PCR analysis using 96-well arrays (or TLDA) after 20-cycle linear preamplification of mRNAs [6].

An alternative to using extracted total RNA is to process directly from cell lysates without any RNA purification step [7]. Indeed, several specific direct cell-based RNA preamplification kits (such as the Takara preamplification kit or kits using specific gene transcript preamplification) can be used with success. We found this procedure has the advantage of recovering 10 to 20-fold more target RNA but has the disadvantage of not permitting directly checking RNA recovery and quality. The genomic DNA is still present in the sample but is not preamplified, so it usually does not give sufficient signal if the probe is still able to recognize some genomic DNA, which is generally avoided whenever possible. Nevertheless, before testing mRNA profiling using such direct cell lysis-based assays, the various steps that are performed for laser capture microdissection (staining procedure in aqueous solutions and microdissection itself) must be checked to ensure they generate limited RNA degradation. This can be easily performed by directly recovering nucleic acids from the whole sections at the end of microdissection with 350 μl of guanidium-based chaotropic solution (RA1 or RP1 from Macherey-Nagel or RLT buffer from Qiagen provided in the extraction kits) with 1% v/v of β-mercaptoethanol. For example, a dozen of coronal mouse coronal sections allows recovering enough total RNA for Nanodrop analysis and RNA 6000 Pico LabChip® Kit analysis. From fresh frozen (snap frozen) rodent tissue, good RNA quality with RIN > 6 (usually 7–9) should be obtained after LCM procedures at least for tissues such as brain/spinal cord or kidney because they contain relative low levels of RNAses as opposed to lung, spleen and even lower for pancreas [8]. Then, it is assumed that the

microdissected cells will provide the same RNA quality as the leftover sections. The collected microdissected cells (100–400 cellular elements dissected in one LCM session) are directly lysed with the Takara lysis buffer in the presence of a RNAse inhibitor at the end of the microdissection procedure as indicated below. We indeed estimated that 200,000–400,000 μm^2 of microdissected cellular elements of interest allow recovering enough RNA (2–5 ng) to analyze 25–100 transcript targets (depending on target abundance) when the mRNAs are preamplified with high specificity (with a 20-cycle preamplification kit such as Takara PreAmpl ver2). If only 5–10 transcript targets are studied, preamplification may not be required depending on their expression level.

2 Materials

1. Use MembraneSlides 1.0 PEN (polyethylene naphtalate) or PET (polyethylene terephtalate) when microdissection is performed at objective ×20, ×40, or ×63. MembraneSlides 0.17 are required when working at objective ×100 (only useful for dissection of subcellular components or very fine processes). PET slides are recommended when using immunofluorescent labeling as for our protocol.
2. For collecting the microdissected cells, we use 0.5 ml tubes with clear caps (AdhesiveCap 500 clear, Carl Zeiss, Germany) that permit checking the effective capture of the cells in the cap. Alternatively, regular 0.5 ml Eppendorf tubes can be used when the cap is filled with guanidium isothiocyanate containing buffer for further RNA extraction.
3. All buffers used for immunolabeling are RNAse-free by using 0.1% DEPC-treated solutions (overnight incubation at room temperature followed by autoclaving). Wear gloves and change them frequently (every 20 min for latex gloves). Use only ready-to-use RNase-free reagents, tubes, and tips.
4. The LCM room containing the laser capture microdissector (PALM MicroBeam, Carl Zeiss, Germany) is equipped with an air-conditioning system keeping ambient air hygrometry below 50% as high humidity is a key detrimental factor for RNA integrity [9]. After use, the LCM room is decontaminated by 30 min UV light exposure.
5. Specific buffers for immunolabeling.

   PBShigh buffer: 10 mM phosphate buffer pH 7.4 (PB) containing high saline (2 M NaCl).

   PBSveryhigh: 10 mM phosphate buffer pH 7.4 (PB) containing very high saline (2.7 M NaCl).

Autoclave these buffers after 0.1% DEPC overnight incubation and aliquot under sterile conditions.

6. RNA purification and reverse-transcription/preamplification is performed in a dedicated room with PCR workstation for RNA work.

3 Methods

3.1 Preparation of Membrane Slides

1. MembraneSlides are usually not guaranteed RNAse-free. The RNase activity can be killed by heating the slides at 180 °C for 4 h. Alternatively, we routinely treat the slides with RNAse-Zap (Thermo Fisher Scientific) or equivalent as follows (**Notes 1** and **2**).
2. Immerge the slide in a 50 ml tube filled with RNAse-Zap (Ambion) for 5 s (*see* **Note 3**).
3. Rinse in a new 50 ml tube filled with RNAse-free water for 5 s, two times.
4. Dry the slides at 37–40 °C for 30 min on a heating plate.
5. MembraneSlides are too hydrophobic to permit correct or easy adhesion of tissue sections to the slides. Irradiate the membrane slides with UV light at 254 nm for 30 min (e.g., in a hood) to make the sections adhere better when sectioning.

3.2 Cryostat Sectioning

1. Central nervous tissue (brain or spinal cord) has been previously fresh-frozen in crushed dry ice or at −45 °C in isopentane and can be kept at −80 °C for up to several months, even a few years. if cutting brains, no freezing medium like Tissue-Tek® O.C.T. compound is required. However, for coronal mouse spinal cord sections, the tissue must be previously embedded in O.C.T. and frozen in suitable plastic molds to ensure future correct positioning. O.C.T. compound will go away in the immunohistofluorescent procedure.
2. All the surfaces in contact with the tissue are treated with RNAse-Zap (Ambion) and rinsed with ethanol. Ten to twelve μm thick sections are prepared using a cryostat at −17/18 °C and thawed onto membraneslides (6–9 sections of mouse spinal cord coronal sections per slide). When the sections are completely dried, RNAses are inhibited and RNA is stable for hours at room temperature. Our method is based on this principle. Ambient humidity below 60% is requested for the day of cutting to prevent rehydration of the sections.
3. Place the RNaseZAP™-treated and UV-treated slides at room temperature.

4. Place a tissue section on the prepared slide. Immediately dry the section with a cold air-hair dryer. Cut another section if required and process as previously.
5. When the desired number of sections is present on the slide, place the slide in a sealed box or a 50 ml tube containing some desiccant. Use the same day or store at −80 °C up to 2 weeks. When the slide is taken out from its −80 °C box, it is immediately air-dried with the cold air-hair dryer to remove any condensed water and place immediately in the LCM room for immunolabeling.

3.3 Immunofluorescent Labeling

1. Draw a water-repellent circle around the section to prevent the waste of reagents (with a PAP pen, or equivalent). Let it dry (*see* **Notes 4–8**).
2. Fix the sections by immerging the slide in a 50 ml tube filled with 96% ethanol, 5 min
   - Remove excess of ethanol and directly place the slide in a 50 ml tube filled with PBShigh buffer, 15 s.
3. (Preincubation). Place the slide on a tray after removing the excess of buffer. Incubate the sections with 100–300 µl of preincubation buffer (75% PBSveryhigh +25% normal donkey serum), 5 min.
4. Replace preincubation buffer by 100–300 µl of PBShigh containing appropriate primary antibodies added just before use, e.g., rabbit anti-GFAP (1/50, DakoCytomation, Glostrup, Denmark) for staining astrocytes and rat anti-mouse CD3ε or CD45 (1/20, BD Pharmingen) for staining immune cells, 10 min incubation.
5. Quickly rinse sections with 100–300 µl of PBShigh buffer.
6. Wash with 100–300 µl PBShigh buffer, 3 min two times.
7. Replace washing buffer by 100–300 µl of PBShigh containing appropriate corresponding secondary donkey antibodies (1/50) added just before use, here AF488-coupled F(ab') 2 anti-rabbit IgG and Rhodamine Red-X or AF594 coupled F (ab')2 anti-rat IgG (Jackson ImmunoResearch, Suffolk, England), 5 min incubation.
8. Quickly rinse sections with PBShigh buffer.
9. Wash with 100–300 µl PBShigh buffer, 3 min two times (*see* **Note 9**).
10. Remove excess of PBS high buffer as much as possible and dip (3 s) the slide in a 50 ml tube filled with 75% ethanol.
11. Immerge the slide in a 50 ml tube filled with 96% ethanol, 3 min.

12. Immerge the slide in a 50 ml tube filled with 100% ethanol, 3 min.
13. Remove excess of ethanol and let the slide dry at room temperature in the LCM room (ambient humidity < 50%) or in a desiccator (*see* **Note 10**).

3.4 Laser Capture Microdissection

The PET-coated slides are placed face up dried directly after staining, and a 0.5 ml Eppendorf tube cap is placed on the dedicated tubing rack of the laser microdissector (*see* **Notes 11–14**).

1. **Drawing cells of interest.** Because the dried CNS sections on MembraneSlides often give nonspecific fluorescence making difficult to visualize the specific signal, it is useful to regularly maintain a film of isopropanol during the drawing step to detect the immunolabeled cells with ease. This is done by adding an isopropanol drop on the area of drawing when needed. In this way, the samples can be processed within 2–4 h after immunolabeling without significant RNA degradation assuming ambient humidity is <50%.

   Undesired cells present nearby target cells can be avoided by stamping AF594-immunolabeled cells in the red fluorescence filter (e.g., immune cells in the EAE white matter, ref. 6). Then on the same field, contours of GFAP-labeled elements are carefully drawn using the chosen adequate Zeiss fluorescent filter set (here FITC filter). 200–400 astrocytic elements (200,000–400,000 μm^2) can be drawn in 30 min to 2 h depending on astrocyte complexity, LCM settings, and experimenter.

   At the end of the drawings and after full evaporation of the isopropanol, astrocytes are microdissected and catapulted either in the 0.5 ml Eppendorf tube cap filled with guanidinium-based lysis buffer (RLT, RNeasy Micro kit, Qiagen, Hilden, Germany) containing 1% β-mercaptoethanol, or directly on the cap from an AdhesiveCap 500 clear tube.
2. **Processing for laser capture.** The cutting/catapulting procedure takes usually up to 30–60 min for 200–400 elements depending on LCM settings and astrocyte complexity. The correct cutting/catapulting is followed on the screen during the entire procedure at ×40 objective under bright light.
   LCM settings for Zeiss PALM (Palm Robo 4.6 software) at ×40 objective are set up the day of experiment. We used LCM settings in the following ranges:

   Cut energy: 26–41; LPC energy delta: 25–90.

   Cut focus: 55–95; LPC focus delta 5–35.

   With 6 μm spot distances, centered.

3. **Collecting cell extracts.** If the cells were lifted in the 0.5 ml Eppendorf tube cap filled with 15–30 µl RLT lysis buffer, centrifuge and store at −20 °C/−80 °C for storage. When the desired number of cell extracts have been collected, the extracts are pooled and completed to 350 µl qsp of RLT (with 1% β-mercaptoethanol) and process for column-based RNA purification.
   If the cells were lifted directly on the AdhesiveCap, remove collecting tube from its tray and add to the cap 10 µl lysis buffer with RT mix and Recombinant RNAse inhibitor as specified in the kit. Collect with the pipette tip as much as the surface inside the cap. Centrifuge the tube and keep on ice before following RNA processing (method A).
   When the microdissected cell lysis extract has been collected, the remaining sections on the PET slide can be extracted with 350 µl RLT + 3.5 µl β-mercaptoethanol for column-based RNA purification (method B).

3.5 RNA Processing

1. Several methods and preamplification kits are available for quantifying target mRNAs by real-time qPCR from trace amounts of initial material. We chose the CellAmp Whole Transcriptome Amplification Kit (Real Time) Ver.2 (Takara, Japan). This kit enables the cDNA (obtained from dT primer-driven reverse transcription) linear amplification (20 cycles) from 200 pg up to 20 ng of RNA per reaction with no nonspecific amplification products derived from the primers. Four reactions can be performed from the initial 5 µl RNA sample (*see* **Notes 15** and **16**).
2. The amplified cDNA (immediately stored frozen) diluted 1/10–1/40 is used as a template for real-time PCR using SYBR and specific primers or better using Taqman probes and adequate real-time PCR mix. Captured cells can be processed by following method A, and if pooled to obtain sufficient RNA (5 ng or more) by following method B. In both cases, left-over sections can be processed following method B to obtain useful information on RNA quality and eventually to provide a corresponding positive control for some gene targets.
3. Method A. This procedure begins the same day of LCM collection starting with 5 µl of extract (cell lysis buffer + RT mix containing RNAse inhibitor) as recommended by the provider (Takara Cat. 3734, Protocol A). Briefly, the extract is transferred to a 0.2 ml PCR tube and incubated at 70 °C for 90 sec and put on ice. After adding MgCl2 and RT Enzyme Mix 2, the cDNA synthesis is performed at 42 °C for 5 min, followed by 5 s at 85 °C in a thermocycler. The sample is centrifuged and stored at −20 °C until next day or at −80 °C for longer time (to process different samples simultaneously). Then, defrost

the samples on ice and heat again 5 s at 85 °C in a thermocycler. Centrifuge and process for exonuclease I reaction, Poly(A) addition reaction and linear mRNA preamplification (reaction 6–9 from Takara's Cat. 3734 Protocol A). Store the preamplified sample at −20 °C.

3.6 RNA Processing with RNA Purification (method B)

1. The RT and cDNA preamplification procedure uses the same kit as method A but starts at a later step (Takara's Protocol B) with the column-based purified RNA (20 ng maximum per 5.6 µl reaction).
2. Without PolyA-carrier RNA use, a minimum of 2.10^6 μm^2 tissue from pooled LCM-collected cells or sections is required for each column for RNA extraction that includes elimination of genomic DNA (NucleoSpin RNA XS from Macherey-Nagel, RNeasy Plus Micro Kit from Qiagen or equivalent).
3. The RNA is eluted in 14 µl water. Two µl are used for RNA analysis via Nanodrop (for tissue sections) or via the Agilent 2100 Bioanalyzer (RNA 6000 Pico LabChip® Kit). The latter analyzes RNA samples with concentrations in the range of 50–5000 pg/µl and provides useful information about RNA quality (degradation/purity) and relative quantity (similar technology is available via other providers). RNA extracts are stored at −80 °C for a limited time (a few days).
4. When all the samples of the same series can be processed, they are pooled and concentrated if required by mild heating (below 65 °C) under a light bulb for several minutes. Then, the RNA (pooled samples if required, 20 ng maximum per reaction) is transferred to a 0.2 ml PCR tube and incubated at 65 °C for 90 s in a thermocycler and put on ice, then processed for reverse-transcription and preamplification as indicated by Takara's Cat. 3734 Protocol B.

3.7 Real-Time qPCR

1. The preamplification product is diluted at least 1/10-1/40 in a 20 µl PCR reaction/well for a 96-well plate-based system.
2. Using SYBR and unlabeled primers, PCR from is performed for 35–40 cycles with fluorescence readings at Tm of the amplicon target minus 3 °C (usually between 74 and 82 °C). Using Taqman probes, PCR is performed for 40 cycles (eventually up to 50 cycles) with fluorescence readings at 60 °C. Under these conditions, no unspecific amplification or residual genomic DNA amplification should be detected. There are now several high-fidelity and high-sensitivity kits for real-time PCR using SYBR green-based detection. For Taqman probes, we use the TaqMan Gene Expression Master Mix (ThermoFisher) that enables detection of small quantities of target (50 pg to 1 ng of cDNA/well) with good accuracy). Both

approaches should permit the detection of housekeeping genes with Ct values comprised between 16 and 22 and of relatively abundant gene transcripts with Ct values comprised between 20 and 35 (threshold for Ct detection setup at 10× background level).

4 Notes

1. General good advices and tips can be found in the booklets "RNA extraction from frozen sections" and "LCM Protocols—RNA Handling "from ZEISS Microscopy Labs Scientific Support, Training and LabService.
2. MembraneSlides (Carl Zeiss, Germany) have a relative short shelf life (1 year maximum at room temperature). A positive effect of UV irradiation of the MembraneSlides is the destruction of potentially contaminating nucleic acids. Note that the sections will still not "jump" on UV-activated MembraneSlides as easily as on positively charged glass slides. This is however sufficient for an experimenter used with cryostat sectioning. Alternatively, brief coating with RNAse-free PolyLysine as used for cell culture dishes may be tested to further facilitate immediate adhesion of the sections to the MembraneSlides.
3. The RNaseZap-treated and UV-treated slides are kept at room temperature and must be used within 2 weeks.
4. The immunolabeling is performed at room temperature just before laser capture microdissection. This procedure uses the PBShigh buffer in all the incubation steps to prevent RNA loss [10]. Any step using physiological saline solutions, water, even 70% ethanol should be avoided or used only few seconds (dip).
5. Do not use nucleic acid stain such as DAPI (4′,6-diamidino-2-phénylindole).
6. Ethanol fixation may be replaced by acetone fixation, but we previously observed poor retention of the sections, specifically for mouse coronal spinal cord sections.
7. Donkey normal serum is used in preincubation buffer since we use secondary antibodies made in donkey. If other species are used, use appropriate corresponding serum.
8. As an alternative to indirect fluorescence immunolabeling (using secondary antibodies), fluorescent labeled primary antibodies can be used to shorten the protocol if required. However, since the short incubation time and the presence of high NaCl in the buffer requires 10–20 times more antibodies than usually required in standard protocols, this approach will be more expensive. Some antibodies cannot withstand the high NaCl concentration.

9. There is no need of RNAse inhibitor in the PBShigh incubation steps as most inhibitors are ineffective at such high saline concentration.
10. We recommend validating the immunostaining procedure on nervous system sections (brain or spinal cord for neural markers) or spleen (for immune cell markers) sections placed on regular glass slides and to check for the preservation of RNA integrity on immunolabeled sections in comparison to unprocessed sections, by extracting RNA from guanidium isothiacyanate based extracts as in B.
11. For the Zeiss PALM LMD system, a last dehydration step with xylene is not required (in contrast to the Arcturus LMD system) though MembraneSlides support up to 10 min incubation with xylene.
12. White matter spinal cord astrocytes are radial glia when there are resting but will become thicker with more GFAP-immunoreactive processes when they become reactive. As we do not stain nuclei with DAPI, we cannot determine precisely the nucleus location and the center of the cell, but proximal GFAP-immunoreactive processes can be easily visualized. These mouse astrocytic elements are 50–100 μm long and 5–20 μm large. Consider that the UV cut will be 1–2 μm large and will destroy the tissue, so draw the line just after the AF488-astrocytic labeling.
13. The LCM settings should be adapted to obtain the most efficient cutting and catapulting settings (differences observed for white matter and gray matter, variations depending on hygrometry, thickness of sections, etc.).
14. Importantly, the experimenter should stay in front of the screen during the whole cutting/catapulting process to eventually correct the focus of the cut as few μm variations may occur when moving the dissecting area. Usually, with the PALM system, the cell is lifted with the first shot but an additional spot inside the drawn element maybe useful (which is covered by setting spot distance closed to 6 μm)
15. The poly(A) RNA carrier (provided in the RNeasy micro kit) may be added only to guanidium-based lysis samples before column-based RNA extraction when RNA quantification, mRNA preamplification, or sensitive downstream applications are not required.
16. Direct lysis buffer method: We here chose Takara's preamplification of mRNA with polydT primers. The advantage of this approach is that preamplified cDNA can be stored and used for a new set of real-time PCR amplification. The inconvenient is that the probes used for real-time PCR have to be within the 1–2 kb of the 3′ of the target mRNA. Probes located more than

2.5 kb of the 3′ end are not efficient. Alternative to this method of amplification, DNAse I treatment of the cell lysate followed by reverse transcription with random primers and specific cDNA preamplification (14 cycles) with the primers for the gene targets to be analyzed, as indicated by Demarest et al. [7]. The advantage of the method is that the location of the real-time primers is not crucial, the inconvenience is that pre-amplified samples cannot be used to analyze a new set of targets. Both the approaches permit the detection of one relatively abundant gene transcript for one or two captured cells, with Ct value comprised between 16 and 35 (threshold for Ct detection setup at 10× background level).

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Chapter 16

Immuno-Laser-Capture Microdissection for the Isolation of Enriched Glial Populations from Frozen Post-Mortem Human Brain

Julie E. Simpson, Stephen B. Wharton, and Paul R. Heath

Abstract

Laser-capture microdissection (LCM) has revolutionized the isolation of defined regions and specific cell populations from human tissue. This approach used in combination with immunohistochemistry (immuno-LCM) has become a valuable method for isolating individual cell-types from a complex heterogeneous population. Here, we describe the detailed methodology required for the isolation of enriched populations of $GFAP^+$ astrocytes, OSP^+ oligodendrocytes, and $CD68^+$ microglia from frozen post-mortem human central nervous system tissue using immuno-LCM.

Key words Immuno-LCM, Rapid immunohistochemistry, Astrocytes, Microglia, Oligodendrocytes, Post-mortem tissue

1 Introduction

Post-mortem human brain tissue, widely available in brain banks, is a valuable resource for the investigation of human neurological diseases. Large-scale gene expression studies, including microarray analysis, have provided valuable insights into pathogenic pathways, but the use of whole brain tissue does not account for the cellular heterogeneity of these samples. While some central nervous system (CNS) cells, such as pyramidal neurons, are easily identified in frozen sections by their morphology using basic histological stains such as toluidine blue, many CNS cell types are less readily identified. There is increasing recognition of the importance of glial cells in the pathogenesis of various CNS disorders, including neurodegenerative and neuroinflammatory diseases, as recently reviewed [1–3]. The ability to identify and isolate glia from frozen human post-mortem CNS material is made possible by combining labeling of specific cell populations using a rapid immunohistochemical

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method combined with laser-capture microdissection (immuno-LCM) [4–7].

Immunohistochemistry uses antibodies against cell-specific markers to identify the cell type. Astrocytes can be identified by expression of glial fibrillary acidic protein (GFAP), an intermediate filament protein [8]. CD68, a lysosomal marker, is commonly used to identify microglia and oligodendrocyte-specific protein (OSP) can be used to label oligodendrocytes [9, 10]. Alternative markers can also be used which may reflect different subpopulations of glia. In human brain, GFAP is upregulated in reactive astrocytes, and will not detect healthy resting populations. A number of other astrocyte markers are available, such as ALDH1, EAAT2, and NDRG2 which may label different populations and for which there is less experience with immuno-LCM [11]. However, an "ideal" marker that can detect all astrocytes in the discrete manner needed for capture is not yet defined and, overall, GFAP currently remains the best available general marker. Similarly, CD68 is upregulated in microglia upon activation. Alternative markers include Iba1, which immunolabels both resting and activated microglia, and MHC class II, which is upregulated in immune-activated microglia [12]. In this protocol, details for GFAP, CD68, and OSP are given.

Immuno-LCM generates samples highly enriched for specific CNS cell types [13]. Although the preservation of a variety of molecular species in human autopsy brain tissue may be affected by variations in post-mortem delay and pre-mortem agonal factors [14, 15], current immuno-LCM protocols can produce cell-type-enriched samples of sufficient quality for gene expression studies [13, 16]. Post-LCM, extracted RNA from frozen material is slightly degraded compared to pre-LCM, but remains of sufficient quality for subsequent downstream analyses [13]. Brain pH can also influence the RNA integrity, as a brain pH of less than 6.0 reduces levels of intact RNA [17]. It should be noted that immuno-LCM produces a cell-type enriched population but, given the intimate relationships between the cell types within the brain and their processes, this is not a pure sample.

Human tissue-based gene expression studies to date have mostly used microarray-based methods. However, the newer RNA deep sequencing approaches to gene expression analysis (RNAseq) have a number of possible advantages over microarray-based methods, including greater dynamic range, sensitivity for low expression differences, and ability to recognize all RNA species [18, 19]. Around 50 ng of total RNA can be obtained from approximately 1000 LCM-ed human glia, adequate as starting material for IVT arrays [13]. Sufficient RNA for RNAseq can be obtained from smaller numbers of cells, and the method is less sensitive to RNA fragmentation than microarrays [20, 21]. Therefore, although there is less experience with RNAseq, immuno-

LCM can provide good cell-type-specific starting material for gene expression studies using either microarrays or RNAseq. Immuno-LCM combined with transcriptomic analysis enables the identification of specific gene expression changes and dysregulation of cellular pathways in different CNS cell types that are relevant to the pathogenesis of neurological disease.

2 Materials

2.1 Immunohistochemistry

1. Uncharged, sterile glass slides (*see* **Note 1**).
2. Acetone at 4 °C (*see* **Note 2**).
3. Tris-buffered saline (TBS): dissolve 6.05 g Tris and 8.76 g NaCl in 800 ml of distilled water (dH_2O), adjust pH to 7.6 with 1 M HCl. Make the volume up to 1000 ml with dH_2O. Autoclave and cool to room temperature prior to use.
4. DEPC water: add 1 ml of 0.1% Diethylpyrocarbonate (DEPC) to 1000 ml dH_2O and mix well. Autoclave and cool to room temperature prior to use.
5. Species-appropriate Vectastain Elite ABC peroxidase kit (Vector Laboratories, UK): 2% normal serum in TBS, 5% biotinylated secondary antibody in TBS, 4% horse-radish peroxidase conjugated avidin/biotinylated enzyme complex (ABC-HRP) in TBS (*see* **Note 3**).
6. Specific glial cell phenotype antibodies (*see* Table 1 for recommended supplier).
7. 4,4′-diaminobenzidine tetrahydrochloride (DAB) (Vector Laboratories, UK).
8. Graded series of alcohol (70% ethanol, 95% ethanol, and absolute ethanol).
9. Xylene.

2.2 Laser-Capture Microdissection

1. PixCell II laser-capture microdissection system (Arcturus Engineering, Mountain View, CA, USA).
2. CapSure Macro LCM caps (Thermo Fisher Scientific, UK).

Table 1
Recommended source and conditions of use of glial specific antibodies

Antibody	Specificity	Isotype	Dilution[a]	Supplier
CD68	Microglia	Mouse IgG_1	1:10	DakoCytomation, UK
GFAP	Astrocyte	Rabbit IgG	1:50	DakoCytomation, UK
OSP	Oligodendrocyte	Rabbit IgG	1:25	AbCam, UK

[a]Antibodies are diluted in the relevant blocking solution

Table 2
Glial-specific primer sequences

Gene	Primer sequence	Product size (bp)
CD68	F: CGA GCA TCA TTC TTT CAC CAG CT	135
	R: ATG AGA GGC AGC AAG ATG GAC C	
GFAP	F: GCA GAA GCT CCA GGA TGA AAC	213
	R: TCC ACA TGG ACC TGC TGT C	
OLIG2	F: CCC TGA GGC TTT TCG GAG CG	474
	R: GCG GCT GTT GAT CTT AGA CGC	

3. Post-it notes.
4. Sterile forceps.

2.3 RNA Extraction

1. PicoPure RNA isolation kit (Thermo Fisher Scientific, UK).
2. Sterile 0.2 ml Eppendorfs.

2.4 RT-PCR

1. Sterile 0.2 ml Eppendorfs.
2. qScript (Quanta, UK) (*see* **Note 4**).
3. Gene specific primers (*see* Table 2 for glial-specific primer sequences).
4. Firepol Green PCR mastermix (*see* **Note 5**).
5. RNase/DNase-free water.
6. 50× Tris-acetate EDTA (TAE) buffer: dissolve 242 g Tris base, 18.61, EDTA and 57.1 ml glacial acetic acid in 1 l dH_2O.
7. 3% agarose gel: 3 g agarose in 100 ml TAE buffer.
8. Ethidium bromide (EtBr,0.5 mg/ml).

3 Methods

Carry out all the procedures using sterile solutions and under RNase-free conditions at room temperature, unless otherwise indicated.

3.1 Rapid Immunohistochemistry for Glial-Specific Markers

1. Freshly prepare and collect 6 μM cryosections from frozen human CNS tissue onto the center of uncharged glass slides (*see* **Note 6**).
2. Allow the sections to warm to room temperature until condensation has completely evaporated from the slides (*see* **Note 7**).
3. Fix the sections in ice-cold acetone for 3 min.

4. Briefly air-dry the sections for 30 s to enable the acetone to evaporate before proceeding.
5. Completely cover the sections with 2% normal serum and incubate for 3 mins.
6. Tap the slides to remove blocking solution (*see* **Note 8**).
7. Apply primary antibody and incubate for 3 min (*see* Table 1 for conditions of use for each recommended antibody).
8. Wash in TBS for 30 s (*see* **Note 9**).
9. Incubate sections with the relevant biotinylated secondary antibody for 3 min.
10. Wash in TBS for 30 s.
11. Incubate the sections with ABC-HRP reagent for 3 min.
12. Wash in TBS for 30 s.
13. Apply the peroxidase substrate DAB and incubate for approximately 3 min (*see* **Note 10**).
14. Stop the reaction by covering the sections with d.H_2O for 30 s.
15. Dehydrate the sections in a graded series of alcohol: 70% ethanol for 15 s, 95% ethanol for 15 s, followed by two immersions in absolute ethanol for 15 s each.
16. Clear the sections in xylene for 30 s.
17. Place the sections in a laminar flow hood to air-dry for approximately 60 min before proceeding to the LCM step (*see* **Note 11**). Representative CD68, GFAP and OSP immunoreactive profiles are shown in Fig. 1.

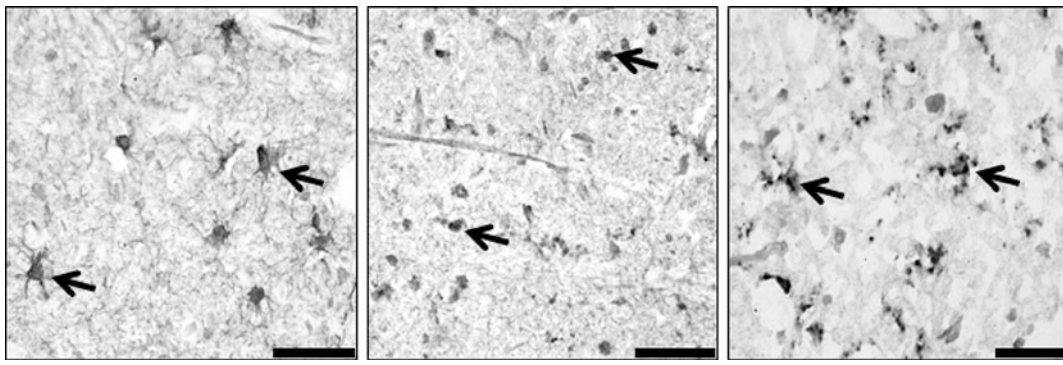

Fig. 1 Rapid immunohistochemical detection of glia cells. (**a**) Glial fibrillary acidic protein (GFAP) immunohistochemistry was used to identify astrocytes, (**b**) OSP to identify oligodendrocytes, and (C) CD68 to visualize microglia. Examples of immunopositive cells are indicated by the arrows. Scale bar represents 50 μm. Reproduced from ref. 13 with permission from Elsevier

3.2 Laser-Capture Microdissection

1. Place a slide on the stage and locate the sample on the microscope/screen.
2. Activate the vacuum to keep the slide in place on the stage.
3. Place the CapSure Macro LCM caps into the slide loader.
4. Move the Pixcell arm across and pick up a cap.
5. Change the size of the laser using lever on the left-hand side of unit, the smallest spot size is 7.5 μM and is recommended for the isolation of human glial cells.
6. Select the laser power (recommended to select 30 mW in the first instance) and duration (recommended to select 15 ms in the first instance) (*see* **Note 12**).
7. Move the cap over the slide and directly lower onto the tissue section.
8. Press laser enable (*see* **Note 13**).
9. Using the joystick, move the laser (seen as a dot on a monitor) over the target cell.
10. Fire the laser (*see* **Note 14**).
11. Repeat **steps 9** and **10** to LCM multiple cells in a view.
12. Raise the cap, turn the vacuum off, move the slide to another area on the tissue, turn the vacuum on, and repeat **steps 9** and **10** until you have microdissected adequate numbers of cells (*see* **Note 15**) (Fig. 2).
13. Raise the cap and remove from the Pixcell unit.
14. Remove any nonspecific tissue from the cap with the sticky strip of a post-it note (*see* **Note 16**).
15. Carefully remove the film from the cap using sterile forceps and place the film in a sterile 0.2 ml Eppendorf.
16. Proceed directly to the RNA extraction.

3.3 RNA Extraction

1. Incubate the film from the LCM cap in 50 μl extraction buffer from the PicoPure RNA isolation kit at 42 °C for 30 min (*see* **Note 17**).
2. Add 250 μl conditioning buffer to a column from the kit.
3. Incubate for 5 min.
4. Centrifuge at 16,000 × *g* for 1 min and discard the flow-through.
5. Add 50 μl 70% ethanol to the sample and mix by pipetting.
6. Add the extract/ethanol solution (approximately 100 μl) to the center of the column (*see* **Note 18**).
7. Centrifuge at 100 × *g* for 2 min.
8. Centrifuge at 16,000 × *g* for 1 min.

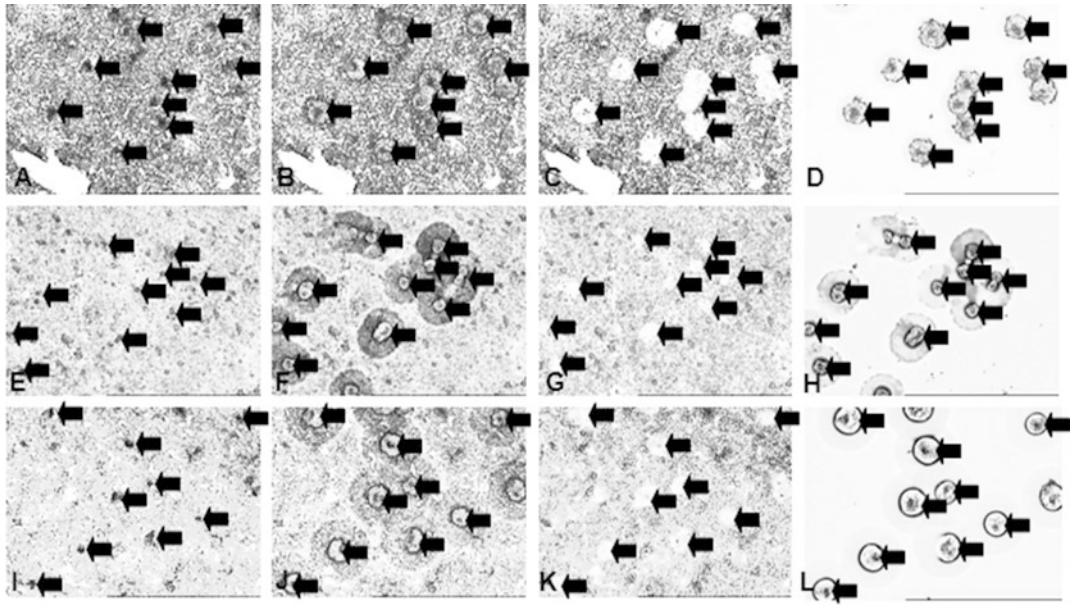

Fig. 2 Laser-capture microdissection of (**a**–**d**) GFAP$^+$ astrocytes, (**e**–**h**) OSP$^+$ oligodendrocytes and (**i**–**l**) CD68$^+$ microglia from frozen post-mortem human brain tissue. Immuno-positive cells were isolated using a PixCell II laser-capture microdissection system. The laser activated the transfer film on a cap placed on the tissue sample, fusing the film with the underlying cell, as indicated by the arrows (**b**, **f**, **j**). The film was lifted off leaving unwanted cells behind (**c**, **g**, **k**). The isolated cells were attached to the film, ready for RNA extraction (**d**, **h**, **l**). Reproduced from ref. 13 with permission from Elsevier

9. Add of 100 µl wash buffer 1 (*see* **Note 19**).
10. Centrifuge at 8000 × *g* for 1 min.
11. Add 100 µl of wash buffer 2.
12. Centrifuge at 8000 g for 1 min.
13. Add 100 µl of wash buffer 2.
14. Centrifuge at 16,000 × *g* for 1 min.
15. Transfer the column to a sterile 1.5 ml Eppendorf.
16. Add 11 µl of elution buffer to the column and incubate for 1 min.
17. Centrifuge at 1000 × *g* for 1 min.
18. Centrifuge at 16,000 × *g* for 1 min
19. Collect the eluant which contains the RNA from LCM-ed cells (*see* **Note 20**).
20. The extracted RNA is now ready for downstream applications, and can be stored at −80 °C until required.

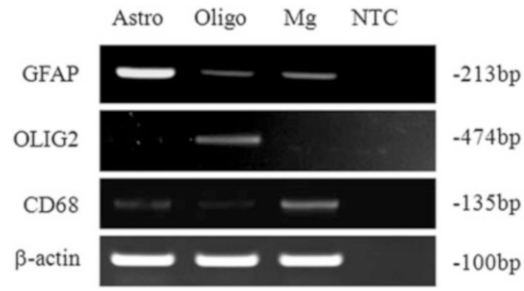

Fig. 3 RT-PCR analysis of glial transcripts in cells isolated by immuno-LCM. $GFAP^+$ astrocytes are associated with high levels of GFAP and low levels of OLIG2 and CD68 transcripts. OSP^+ oligodendrocytes are associated with high levels of OLIG2 and low levels of GFAP and CD68 transcripts. $CD68^+$ microglia are associated with high levels of CD68 and low levels of GFAP and OLIG2 transcripts. Key: Astro, astrocytes; Oligo, oligodendrocytes; *Mg* microglia, *NTC* no template control, *GFAP* glial fibrillary acidic protein. Reproduced from ref. 13 with permission from Elsevier

3.4 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Analysis of Glial Cell Enrichment

1. Synthesize cDNA using qScript cDNA mix/supermix, according to the manufacturer's instructions.
2. Into a sterile 0.2 μl Eppendorf place 50 ng cDNA, 5× Firepol Green and optimized concentrations of forward and reverse glial-specific primers in a total volume of 20 μl (*see* Table 2).
3. Following denaturation at 95 °C for 10 min, amplify the PCR products using the following thermal cycle: for CD68 and GFAP (40 cycles at 95 °C for 15 s and 60 °C for 60 s) and for OLIG2 (35 cycles at 95 °C for 60 s, 60 °C for 45 s and 72 °C for 60 s), then 72 °C for 15 min.
4. While the PCR is running prepare a 3% agarose gel, microwaving on full power to ensure all the agarose melts, thereby avoiding particles in the gel (*see* **Note 21**).
5. Cool the gel by running cold tap water over the surface of the flask.
6. Add 1 μl EtBr to the cooled gel mix, pour the gel into an appropriately sized casting tray with comb and leave the gel to set (*see* **Note 22**).
7. Visualize the PCR products on the 3% agarose gel stained with ethidium bromide, ensuring the PCR products are the correct predicted molecular weight (*see* Table 2 and Fig. 3).

4 Notes

1. Uncharged glass slides enable the detachment and isolation of specific cells from the tissue during microdissection. Tissue sections adhere more strongly to charged slides and will require the

laser power and/or duration to be increased to enable specific cells to be isolated, potentially causing RNA degradation.

2. Ice-cold acetone is used to fix the tissue sections to the slide to avoid detachment of the tissue during the immunohistochemistry staining step. Methanol can be used as an alternative, but paraformaldehyde should be avoided as this cross-linking reagent results in a significant decline in the quality of RNA extracted from the tissue.
3. Use the appropriate kit depending on the species that the primary antibody is raised in: in the current protocol the Vectastain Elite ABC peroxidase mouse kit is used to detect CD68 and Vectastain Elite ABC peroxidase rabbit kit for GFAP and OSP. The ABC-HRP component of the kit must be made 30 min prior to use.
4. While alternative techniques to synthesize cDNA can be used, the qScript cDNA SuperMix is a sensitive and reliable one-step reagent for first-strand cDNA synthesis.
5. Alternative PCR mastermixes can be used. The mixes that include a loading buffer ease the process of running the products on the agarose gel.
6. In our hands, preparing the cryosections immediately before use reduces RNA degradation.
7. It is essential to allow the sections to warm to room temperature as the tissue will detach from the slides if they are cold when you immerse them in the acetone.
8. No wash is required at this stage as the primary antibody is made in the blocking solution.
9. All the wash steps throughout the protocol are crucial to remove any weak attachment of the antibody, and reduce nonspecific background staining.
10. The 3 min application of DAB is a guide. It is recommended that you watch the reaction develop under the microscope, where the antibody has bound to the antigen will be visible as brown staining. When the cell population of interest is visible, immediately place the sections in d.H_2O to quench the reaction.
11. Complete dehydration of the sections after the rapid immunostaining stage is critical to enable the isolation of good quality RNA.

12. The power and duration of the laser can be increased if these settings do not melt the film on the LCM cap. However, to avoid unnecessary additional RNA degradation select the lowest power and duration required to microdissect the cells of interest.
13. For safety, the laser will only come on if the cap is correctly positioned on the slide.
14. Fire the laser once; multiple firings will take more nonspecific tissue along with the cell of interest.
15. We recommend the LCM isolation of approximately 1000 $GFAP^+$ astrocytes, 1000 $CD68^+$ microglia, and/or 1500 OSP^+ oligodendrocytes to obtain around 50 ng total RNA, which is adequate for subsequent microarray or RNAseq analysis.
16. The sticky surface of a post-it note is sterile and will remove the weakly attached nonspecific tissue which may have attached when the cap was raised. Only the specific LCM-ed cells will remain.
17. Following this stage the sample can be safely stored at –80 °C before continuing.
18. Take care to gently apply the solution to the center of the filter in the column, without touching or damaging the filter.
19. This series of stringency washes is essential to remove any weakly bound non-RNA material present in the sample.
20. At this stage we strongly recommend both the quantity and the quality of the extracted RNA are determined using a Nanodrop 1000 spectrophotometer (Thermoscientific, UK) and 2100 Bioanalyzer RNA 6000 Pico LabChip (Agilent, UK), respectively. The Bioanalyzer Picochip will assign an RNA Integrity Number (RIN) to your sample which is an indication of the quality of your extract. A RIN of 10 indicates fully intact RNA, whereas a RIN of 1 indicates totally degraded RNA. RIN numbers of between 2 and 3 are normal for LCM-ed post-mortem human samples, but in our hands are of sufficient quality for downstream applications. Representative RNA profiles pre- and post-LCM are shown in Fig. 4.
21. Ensure care is taken as the gel will be very hot.
22. EtBr intercalates DNA making it visible under UV light. It is a potent mutagen, so take extra care to ensure you do not come into direct contact with it.

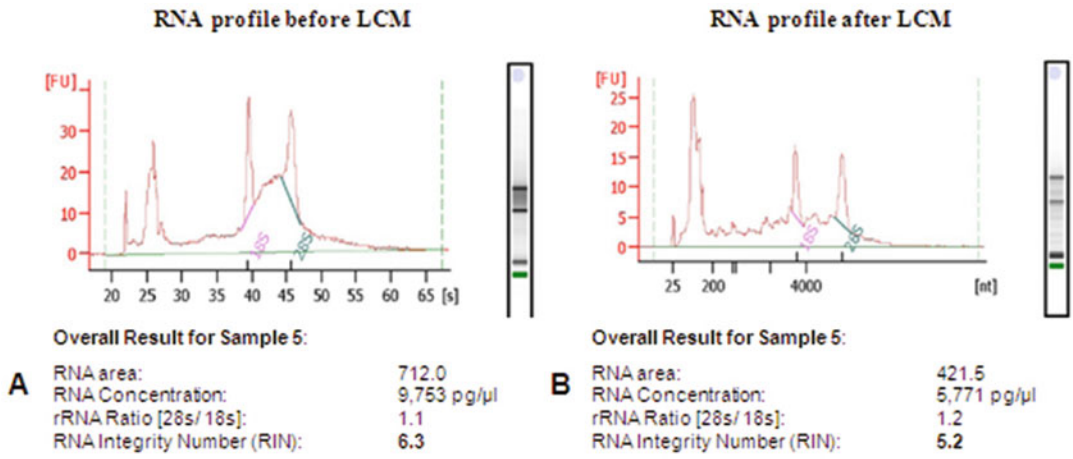

Fig. 4 Laser-capture microdissection of immuno-positive cells from post-mortem CNS is associated with a decrease in the RNA integrity number. (**a**) The RIN value of 6.3 pre-LCM, decreases to (**b**) RIN 5.2, post-LCM. Reproduced from ref. 13 with permission from Elsevier

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Chapter 17

Laser-Capture Microdissection for the Analysis of Rat and Human Spinal Cord Ependyma by qPCR

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Abstract

In the last few decades many efforts have been dedicated to decipher the nature and regenerative potential of neurogenic niches and endogenous stem cells after damage of the central nervous system. In the spinal cord, it has been largely focused on the ependymal region, which hosts neural precursors/stem cells (NSC) in rodents but differs between species and ages. In the current chapter, we detail our protocol to study the gene expression profile of this region using fresh frozen blocks of rat and human post-mortem spinal cords. We describe how to prepare and process those tissues, how to identify and dissect the ependymal region using Laser-Capture Microdissection (LCMD), and how to isolate and amplify RNA with different integrity states to finally obtain enough material for performing gene expression assays using Taqman® Low Density Arrays. LCMD technique maintains tissue integrity allowing for subsequent analysis without manipulation steps that may alter molecular properties of cells and the eventual loss of delicate cell types in comparison with other approaches that require previous disaggregation of the tissue and cell manipulation before isolation.

Key words Laser-Capture Microdissection, Neural stem cells, Spinal cord, Interspecies, Ependymal, Neurogenesis

1 Introduction

Current advances in molecular biology and the widespread use of powerful genetic tools have extended to many laboratories the capacity of performing comparisons between gene expression profiles. Those analyses may allow the identification of key molecular players differentially activated in anatomical regions or cell populations in different conditions such as development, aging, health, and disease. However, the heterogeneity of cell types present in almost any tissue advises on the use of procedures to previously purify or, at least, enrich the sample with a particular cell population to facilitate data interpretation. This can be achieved by approaches

Graeme I. Murray (ed.), *Laser Capture Microdissection: Methods and Protocols*, Methods in Molecular Biology, vol. 1723, https://doi.org/10.1007/978-1-4939-7558-7_17,

that maintain tissue integrity before isolation like Laser-Capture Microdissection.

Laser-Capture Microdissection (LCMD) consists in extracting pieces of tissue from slices or culture dishes after cutting them with a laser mounted on a microscope device. LCMD entails the identification of the desired cell population prior to cell separation but then allows for subsequent analysis without manipulation steps that may alter molecular properties of cells and produce the eventual loss of delicate cell types. The identification of the desired cells can be made by location, morphology (using general histological staining), the expression of fluorescent markers or by quick immunostaining protocols.

There are two general strategies for LCMD: (1) Infrared-capture systems (IR), in which a near-IR laser is used for melting a thermolabile polymer film that fuses with the tissue to form a complex later recovered for nucleotides isolation; (2) UV cutting systems, in which a pulsed UV-A laser is focused through lenses down to a small spot size producing enough energy to rapidly cut off the irradiated material avoiding the transfer of heat into the surroundings. Non-focused laser light becomes scattered and travels through adjacent areas without impact on the specimen. The wavelength of the applied UV laser (337 nm in our case) is sufficiently far away of the peak of absorption of DNA, RNA, or proteins and does not interfere with the genome or proteome [1–4].

After cutting, tissue can be recovered without manipulation. For this, some systems are based on gravity, in which the sectioned cell/region drops in the cap of a tube, while others are based on "catapulting" the sample by the photonic force produced by a defocused UV laser pulse [1–4]. In the current protocol, we use a LEICA DM6000 laser microscope, based on gravity-assisted microdissection, in which laser vaporizes a polyethylene glycol-based UV-absorbing membrane (polyethylene naphthalate or PEN) that supports tissue sections, causing that dissectate drops into the cap of a microcentrifuge tube.

As discussed in the chapters of this book, the source for RNA can be multiple and there are kits and protocols useful to isolate RNA, DNA, and proteins from both frozen or formalin-fixed, paraffin-embedded (FFPE) material. However, fresh frozen tissue is preferred for isolating RNA, especially if frozen immediately after surgery. This, together with precautions taken during the procedure (like performing it in cold and as quickly as possible), minimizes RNA degradation by RNases.

In the current chapter, we will show the protocol that we use to study gene expression profile of the ependymal region of the spinal cord. In our group, we are interested in deciphering the nature and regenerative potential of neurogenic niches and endogenous stem cells after spinal cord damage. Specifically, we have focused on

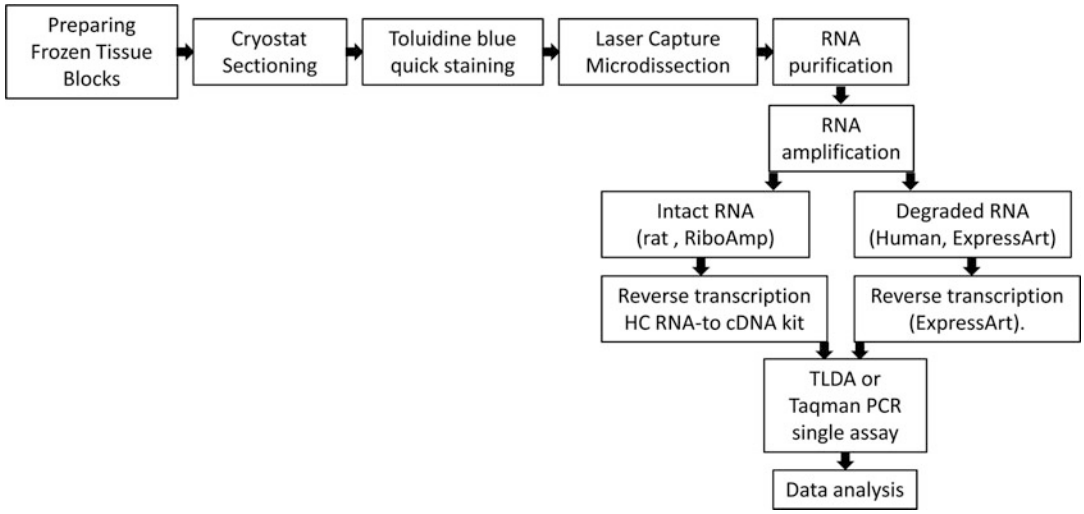

Fig. 1 Workflow of the procedures included in the current chapter

characterizing the ependymal region of the spinal cord, which hosts neural precursors/stem cells (NSC) in rodents [5–7]. Even though some works have already been published on the topic, the true nature of NSC and their markers, the changes in their properties along the lifespan of animals, and the extent of the differences in this niche between rats and humans are still a matter of debate [6–12]. To contribute to the discussion, we have studied gene expression profiles of ependymal regions from rats and humans, using fresh frozen human post-mortem spinal cord tissue from Spanish Public Tissue Banks, and prepared their equivalent fresh frozen rat spinal cord tissue. In the following protocols, we describe how we prepare and process those tissues to finally obtain enough RNA that can be tested by Taqman® qPCR. This procedure has been successfully used and reported in our previous publications [10, 11, 13].

The workflow comprises several processes (*see* Fig. 1): rat surgery and freezing protocols (or recovery of human samples), cryosectioning, toluidine blue staining for ependymal cell visualization, Laser-Capture Microdissection, RNA extraction, quantification and quality check, RNA amplification, retrotranscription, and Taqman Low Density Array assays.

2 Materials

2.1 Sample Preparation

RNA surface cleaning product (RNAse Zap).
Sodium Pentobarbital.
1 cc syringe.
25 G 1½″ orange needle.

Sterile surgical instruments.
Cold sterile Saline solution: NaCl (0.9%) in sterile water.
Razor blade.
Cryo-tubes 2 ml.
Dry ice.
Cryostat.
Acetone.
Disposable cryostat blades.
Optimal Cutting Temperature compound (OCT-compound).
PEN-Slides.
Sterile 50 ml Falcon (one per PEN-slide).
Desiccating silica gel (1 g packets).

2.2 Toluidine Blue Staining

DEPC water: Water treated with 0.1% DEPC for 12 h at 37 °C, then heating to 100 °C for 15 min or autoclaving for 15 min to break down the DEPC.

Ethanol (EtOH) 70% in DEPC Water (25 ml for a 5-slides staining jar/bucket).

EtOH 100% DEPC Water (25 ml for a 5-slides staining jar/bucket).

Toluidine-DEPC: Dissolve 0.15 g of toluidine blue poder in 15 ml of DEPC water. Mix with vortex and filter by sterile filter (15 ml is enough for a 5-slides staining jar/bucket).

Staining jars/buckets (5-slides).

2.3 Laser-Capture Microdissection

Sterile 50 ml Falcon (one per PEN-slide).

0.5 ml RNase-free Eppendorf tubes (one per PEN-slide, one additional for debris collection).

mRNA extraction buffer (included in the Arcturus PicoPure RNA Isolation Kit, Applied Biosystems).

Microcentrifuge.

Dry ice.

2.4 RNA Extraction and Amplification

Thermocycler with heated lid.

Microcentrifuge for 1.5 and 0.5 ml tubes (must reach 16,000 × *g*).

0.5–10 μl pipette.

20 μl pipette.

200 μl pipette.

1000 μl pipette.

Ice bath or cold block (4 °C).

0.5 or 0.2 ml RNase-free microcentrifuge tubes.

2 ml lidless tube for centrifuge.

Nuclease-free pipette tips.

Arcturus® PicoPure® RNA isolation Kit (Applied Biosystems, Lithuania).

DNase I treatment (QIAGEN RNase-Free DNase Set).

Experion™ Automated Electrophoresis Station.

Experion Priming Station.
Experion™ Vortex Station II.
Experion Software.
Experion™ RNA HighSens kit.
RNA Amplification kits: For Rat mRNA or tissues with good RNA integrity, use 2-round Arcturus RiboAmp® PLUS RNA Amplification Kit (Applied Biosystems, USA); For human mRNA or tissue with poor RNA integrity use 2-round ExpressArt® TRinucleotide mRNA Amplification Kit (AmpTec, AMSBIO).
Experion™ RNA StdSens kit (BioRad, USA) or Nanodrop spectrophotometer (Nanodrop, Thermo Scientific, USA).

2.5 Retro-transcription and Taqman Low Density Arrays

ExpressArt_TR cDNA synthesis kit (AmpTec, AMSBIO).
TaqMan® Universal Master Mix II (Life Technologies, Madrid, Spain).
Taqman Low Density Arrays (TLDAs; Life Technologies, Madrid, Spain).
Applied Biosystems 7900HT Fast Real-Time PCR System.
Thermocycler.
Pipette-tips (filter-tips recommended).
Reaction tubes (PCR tubes/1.5 ml).
100% Ethanol and 70% Ethanol.
Microcentrifuge.

3 Methods

During all the procedures, it is advised to wear gloves and work in RNase-free conditions: If possible, dedicate a specific place in the laboratory, clean surfaces with RNase Zap or equivalent products, use RNase-free tubes and media, and treat water or solutions with DEPC (*see* **Note 1**). It is recommended to work as quickly as possible for obtaining the best RNA quality; therefore, please set the material and devices ready before starting protocol: Irradiate dissection material and PEN slides with UV light for 30′ (*see* **Note 2**), clean cryostat knifes with acetone, clean the cryostat and laser microscope stages with RNAse Zap, and let the dissecting microscope ready (clean, turned on, lasers aligned and the appropriate settings loaded).

3.1 Sample Preparation

This procedure shows how to obtain samples from experimental animals to finally get frozen sections on slides. When working with frozen tissue blocks as starting material, the protocol can be followed from Subheading 3.1.2.

3.1.1 Obtaining Tissue Blocks and Slices (Fig. 2)

1. Mark cold cryotubes: 3 per rat (for collecting cervical, thoracic, and lumbar segments).
2. Euthanize rat with sodium pentobarbital overdose (200 mg/kg body weight).

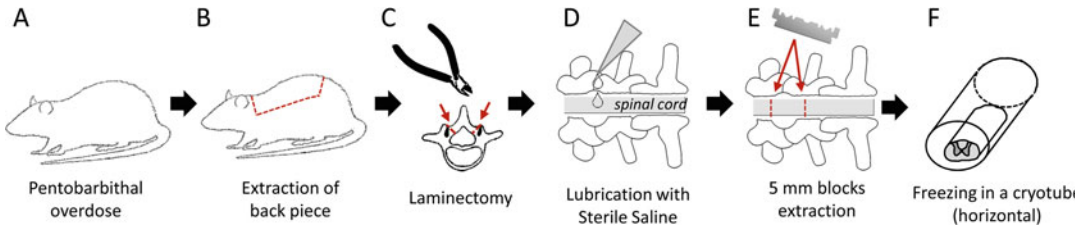

Fig. 2 Scheme depicting the procedure for spinal cord extraction from rats. After pentobarbitate overdose (**a**), skin muscles and bones are cut according to the *red lines* and back piece is extracted (**b**). After the paraspinous muscles are separated from the vertebra, laminae can be cut with a bone cutter or a wire side cutter following the *red lines* as depicted (**d**). Then, lubrication with sterile cold saline is advised (**e**) before cutting 5 mm pieces with half razor blade (**f**). Spinal cord blocks are freezed in cryotubes in horizontal position (**f**). Care must be taken not to deform spinal cord during the process

3. Once animal is dead, cut off head using Mayo scissors, open the skin of the back from the neck to the waist, holding skin with tissue forceps. Cut muscles, spine, and spinal cord just above the waist. Laterally cut muscle and ribs and separate viscerae from the ventral spine using a blunt dissection.
4. Once the back piece is separated, it can be easily handled to longitudinally clean the paraspinous muscles from the vertebral processes with scissors or scalpel blade and to cut and remove the dorsal vertebral arch. For this a micro-rongeur or a bone cutter can be used (*see* **Note 3**).
5. Once the spinal cord is dorsally exposed, cut 5 mm pieces (cervical, thoracic, and lumbar) with a disposable razor blade (using half blade). It is very important to avoid spinal cord deformation during extraction for a subsequent easy identification and isolation of ependymal region. For that, be extremely gentle when extracting spinal cord, and lubricate pieces before extraction pouring cold sterile saline on top of them.
6. Extract pieces without deforming them with sterile Iris forceps and place gently each piece in a cryotube in the horizontal position. Then, freeze the tube and the cord with dry ice.
7. Store at −80 °C until use.

3.1.2 *Cryostat Sectioning*

1. Set cryostat at −23 °C.
2. Adhere spinal cord blocks to the cutting stage with some drops of OCT (do not embed the whole block in OCT, *see* **Note 4**).
3. Cut 25 μm thick slices and Collect them on PEN slides maintained at room temperature (Fig. 3) (*see* **Note 5**).
4. Once filled with slices, temper slide 2′ at RT in a 50 ml Falcon tube filled with dissecting packets. Place packets only in the back side of the slide to avoid tissue damage (*see* **Note 6**).

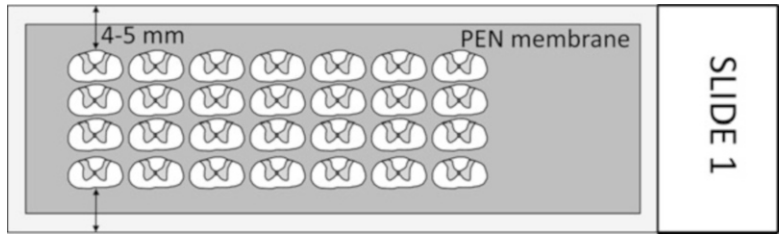

Fig. 3 Drawing representing the ideal placing of cryosections on the PEN slide. A 4–5 mm tissue free gap should be used since microscope range may not reach the limits of the pen membrane (*dark gray*)

3.1.3 Toluidine Blue Staining

Process slides one by one. The whole staining should be performed on ice, in UV treated buckets.

1. Take off the slide from the tube and dry it 10′ at 40 °C in a stove.
2. Immerse the slide in 70% EtOH for 1 min (quick fixation).
3. Quick rinse by carefully dipping slide in DEPC-water (5 dips).
4. Immerse the slide in toluidine blue solution for 2 min.
5. Two quick rinses in DEPC-water (about 15 s each).
6. Differentiate in 70% EtOH, 1 min.
7. Differentiate in 100% EtOH, 30 s.
8. Drain liquid excess on a filter paper by vertically holding slide and then completely dry it at 40 °C in a stove for 15 min.
9. Total time accumulated at this point (Subheadings 3.1.2 and 3.1.3): 45 min, approximately.

3.2 Laser-Capture Microdissection

Prepare a microscope before starting the experiment to reduce the duration of the tissue processing.

3.2.1 Microscope Preparation (LEICA LMD-6000)

1. Clean the stage and pieces that will be in contact with the slide using RNAse Zap or other surface cleaning solutions.
2. Perform focusing with the objectives you will use and laser alignment according to the microscope user's manual. For this, a RNAse-free tube with 50 µl of DEPC-water can be used to collect discarded pieces while laser focusing and alignment. Follow the user manual of each specific microscope for this procedure.
3. Since microdissection is performed without coverslips or immersion oils, tissue sections show refraction-derived distortions in the image when completely dried, which can make it difficult to distinguish the cells of interest. This can be improved using a light diffuser in the illumination path.

3.2.2 Laser Microdissection

1. Slides can be transported to the microscope inside a sterile/RNAse-free 50 ml falcon tube (*see* **Note 7**).

Table 1
Laser settings for cutting 20–25 μm slices

	10× objective	40× objective
Laser power	60	55
Aperture	10	3
Laser speed	15	17
Offset	60	90

2. Place a 0.5 ml tube in the tube holder, adjusting the cap in the proper slot. Place the tube holder into stage support and carefully fill the cap with 50 μl of Pico Pure extraction buffer.
3. Unload specimen holder and place the slide on one of the holder holes.
4. Focus the sample using low magnification (6.3×) and search for central canal or ependymal region.
5. Increase magnification to 40×.
6. Draw shape. It is important to draw it close to the basal part of the central canal cells nuclei but without touching them. This allows recovering most of the cuttings and collecting only the cells in the ependymal layer. Leaving a large gap from nuclei greatly lowers the efficacy of cutting, since adjacent parenchyma contains regions where laser burns the tissue without cutting (*see* **Note 8**).
7. Use the laser settings for 20–25 μm slices according to Table 1.
8. After cutting the ependymal region in all the slices, close the cap of the eppendorf tube and incubate it lying on its cap for 30 min at 42 °C in a stove.
9. Centrifuge for 2 min at 800 × *g* (4 °C). If centrifuge does not include small holes, introduce the small eppendorf tube on a cap-less RNAse-free 2 ml tube as support.
10. At this point, the solution can be frozen and maintained at −80 °C until use.

3.3 RNA Extraction

1. We use the Arcturus® PicoPure® RNA isolation Kit, according to the manufacturer's instructions. The following paragraphs have been mostly elaborated by directly quoting from the manufacturer's protocol:
2. Pre-condition the RNA Purification Column by pipetting 250 μl Conditioning Buffer (CB) onto the purification column filter membrane and incubate the column for 5 min at room temperature. Centrifuge the column in the provided collection tube at 16,000 × *g* for 1 min.

3. Pipette 10 µl of 70% Ethanol (EtOH) into the cell extract, mixing well by pipetting up and down. Pipette the mixture into the preconditioned purification column (approximate volume of 20 µl) and centrifuge for 2 min at 100 × *g* to bind RNA. Centrifuge immediately at 16,000 × *g* for 30 s to remove flow-through.
4. Pipette 100 µl Wash Buffer 1 (W1) into column and centrifuge for 1 min at 8000 × *g*.
5. Before further washing and final elution, we treat samples with DNAse I to eliminate genomic DNA: Pipette 5 µl DNase I Stock Solution to 35 µl Buffer RDD (provided in the Set). Mix by gently inverting and pipette the 40 µl DNase incubation mix directly into the purification column membrane. Incubate at room temperature for 15 min and then pipette 40 µl PicoPure RNA Kit Wash Buffer 1 (W1) into the column, centrifuging then at 8000 × *g* for 15 s.
6. Pipette 100 µl Wash Buffer 2 (W2) into the purification column and centrifuge for 1 min at 8000 × *g*. Then pipette another 100 µl Wash Buffer 2 (W2) into the column and centrifuge for 2 min at 16,000 × *g*. Check the purification column for any residual wash buffer. If wash buffer remains re-centrifuge at 16,000 × *g* for 1 min.
7. Transfer the purification column to a new 0.5 ml microcentrifuge tube provided in the kit and pipette 11 µl of Elution Buffer (EB) directly onto the membrane of the purification column (gently touch the tip of the pipette to the surface of the membrane while dispensing the buffer).
8. At this point, the RNA solution can be frozen and maintained at −80 °C until use. In this case, we recommend separating a 2 µl aliquot for RNA quantification and evaluation of purity and integrity by high sensitivity electrophoresis techniques (*see* **Note 9**).

3.4 RNA Analysis

1. For RNA quantity and integrity evaluation we use chip-based electrophoresis (BioRad Experion™ RNA analysis kits), to perform reproducible and quantitative analyses. At this stage, Experion™ RNA HighSens kit should be used, since LCMD renders picograms of RNA.
2. Experion protocol is performed according to the manufacturer's instructions. The following paragraphs (except for the notes) have been elaborated mostly by directly quoting from the manufacturer's protocol.
3. *Experion Station preparation*: Clean electrodes. Place a cleaning chip filled with 800 µl Experion electrode cleaner into the electrophoresis station. Close the lid for 2 min; remove the cleaning chip. Place another cleaning chip filled with 800 µl

DEPC-treated water into the electrophoresis station. Close the lid for 5 min. Replace the water in the cleaning chip and repeat the rinse step. Remove the cleaning chip, and leave the lid open for 60 s to allow the electrodes to dry completely.

4. *Buffer Preparation*: Remove one tube of each kit reagent from storage, place the RNA ladder (red cap) on ice, and allow all other reagents to warm to room temperature (~15 min). Briefly vortex and then centrifuge the tubes before use to collect the solutions at the bottom of the tubes. Protect stain from light at all times. Then Prepare the Gel and Gel-Stain Solution. Centrifuge 600 μl RNA gel (green cap) in a spin filter at 1500 × *g* for 10 min (unused filtered gel may be stored at 4 °C for 1 month). Pipet 65 μl filtered gel into an RNase-free microcentrifuge tube; add 1 μl RNA stain (blue cap) to the filtered gel and vortex. Centrifuge gel-stain solution at 13,000 × *g* for 10 min. Prepare the Samples and RNA Ladder. Pipet at least 5 μl RNA ladder (red cap) and at least 2 μl each RNA sample into RNase-free microcentrifuge tubes; denature the samples and ladder for 2 min at 70 °C. For the RNA ladder, use a 1.5 ml tube (*see* **Note 10**).
5. Immediately place the samples and ladder on ice; leave them on ice for 5 min. Briefly spin down the tubes and then mix; store on ice. Add 795 μl DEPC-treated water to the tube of 5 μl denatured RNA ladder and vortex.
6. Aliquot denatured, diluted RNA ladder. Store one tube on ice and immediately freeze remaining aliquots on dry ice and then store at −70 °C.
7. *Prime the Chip*: Remove an RNA HighSens chip from its packaging and place it on the chip platform in the Experion priming station. Add 9 μl gel-stain solution to the gel priming well. Close the lid of the priming station, set the pressure to B, and set the time to 1. Press the Start button. When priming is complete, remove the primed chip from the priming station. Flip the chip over, and visually inspect the microchannels for trapped air bubbles or incomplete priming (*see* **Note 11**).
8. Load the Samples and RNA Ladder Into the Chip: Pipet 9 μl of gel-stain solution into the other well-labeled GS, 9 μl of filtered gel into the well-labeled G, 6 μl of sensitivity enhancer (clear cap) into the well-labeled SE, and 5 μl of loading buffer (yellow cap) into each sample well (1–11) and into the well-labeled L (ladder well); do not leave any sample well empty. Pipet 1 μl denatured, diluted RNA ladder into the well-labeled L. Pipet 1 μl denatured sample into each of the 11 sample wells; pipet 1 μl DEPC-treated water into any unused sample wells. It is advised to pipette volumes slow but continuously, close to the bottom of each well to avoid bubbles.

9. Place the chip in the Experion vortex station and vortex for 1 min. Run the chip in the Experion electrophoresis station within 5 min of loading.
10. Place the chip on the chip platform of the electrophoresis station and close the lid. Select New Run, then the Total RNA assay (*see* **Note 12**). Click the Start button on the screen to begin the run. When the run is complete, remove and discard the used chip. Clean the electrodes placing a cleaning chip filled with 800 μl DEPC-treated water into the electrophoresis station. Close the lid for 60 s to clean the electrodes, and then open it for 60 s to allow the electrodes to dry completely. Remove the cleaning chip.
11. *Data analysis*: At the end of each run the Experion software will display an electropherogram of the RNA ladder, and generates nine peaks. The software will identify the first peak as the lower alignment marker. If the RNA separation was successful, the other eight RNA peaks in the ladder should be well resolved. The two most prominent peaks represent 18S and 28S ribosomal RNA (rRNA). Experion Software shows then the quantity and a RNA Quality Score: RNA Quality Indicator (RQI). RQI calculations are based on mapping an RNA sample's electropherogram profile into a set of degradation standards, in which ribosomal RNAs degradation and modifications in different regions of the electropherogram are measured. Software returns a RQI number between 10 (intact RNA) and 1 (highly degraded RNA) for each sample, accompanied with a color code (green color for samples above 7, yellow color for samples with RQIs from 4 to 7, and red color for samples below 4) [14] (*see* **Note 13**).
12. In our experiments, 100–200 ependyma were collected per rat, obtaining 5–50 ng of total RNA and RQI are normally above 7 (green color) and are not considered when scoring RQIs < 6. In humans, 30–70 ependyma were collected per individual, obtaining 15–150 ng of total RNA. Human spinal cord samples are usually not in the best conditions and average RQIs are normally in the yellow interval. Although all the samples can be amplified with the specific kit (*see* below), we have not reported results using the samples with red RQI (i.e., below 4).

3.5 RNA Amplification

RQI scores of rat samples showed that RNA quality was good and the rest of the workflow was performed using popular linear amplification kits (2-round Arcturus RiboAmp® PLUS RNA Amplification Kit). However, human samples showed very low RQIs and were submitted to an amplification method specifically developed designed for amplification of mRNAs or mRNA fragments without poly(A) and for severely degraded eukaryotic RNAs (2-round ExpressArt® TRinucleotide mRNA Amplification Kit; [15]) (*see* **Note 14**).

3.5.1 Arcturus RiboAmp® PLUS RNA Amplification

The RiboAmp® PLUS RNA Kit achieves high yields of aRNA with linear amplification using double-stranded cDNA as a template in a T7 RNA polymerase catalyzed amplification. *The following paragraphs have been mostly elaborated by directly quoting from the manufacturer's protocol*:

1. Do not overload amplification reactions. Choose the appropriate kit according to the total RNA input requirements (*see* **Note 15**). In our experiments, we have used Riboamp PLUS 2-round.
2. This is a five-step process for linear amplification: (1) first strand synthesis reaction that yields cDNA incorporating a T7 promoter sequence; (2) second strand synthesis reaction utilizing exogenous primers that yields double-stranded cDNA; (3) cDNA purification with Columns; (4) In vitro transcription (IVT) utilizing T7 RNA polymerase yields antisense RNA (aRNA); (5) aRNA isolation with purification columns (*see* **Note 16** for protocol general advices).
3. Prepare reagents: Thaw the frozen kit components as needed, and mix by flicking the tube or by inverting the tubes several times, spin down, and place on ice. Allow In Vitro Transcription (IVT) Buffer (Blue-labeled Vial 1), Master Mix (Blue labeled Vial 2), and Enhancer (Yellow-labeled Vial) to assume room temperature, and mix by inverting or flicking the tube. Spin down if necessary. Dissolve all visible solids prior to use.

Round One: first strand cDNA synthesis

4. After Picopure RNA extraction, the RNA sample should represent a total volume of 9–10 μl in a 0.5- or 0.2-ml RNase-free microcentrifuge tube (11 μl of eluted samples minus the 2 μl used for Experion quantification). Place them on 4–8 °C block.
5. Program Thermal cycler according to Table 2.
6. Add 1 μl of Primer A (Grey-A vial), mix well, and spin down.
7. Incubate at 65 °C for 5 min and then chill the samples to 4 °C for at least 1 min. Hold the sample at 4 °C until ready to proceed.
8. Spin the contents down before proceeding to the next step.
9. Thaw First Strand Synthesis components (Red-labeled Vials) and place on ice.
10. Add reagents directly to the sample: 7 μl of First Strand Master Mix (Red-1 vial); 2 μl of First Strand Enzyme Mix (Red-2 vial); Total volume per sample 9 μl.
11. Incubate at 42 °C for 45 min, and then chill the sample to 4–8 °C for at least 1 min. Do not hold the samples at this step for a prolonged period of time. Keep the samples at 4–8 °C until next incubation.

Table 2
Thermocycler programming for Arcturus RiboAmp Round One

	°C	Time
First strand synthesis	65	5 min
	4	Hold
	42	45 min
	4	Hold
	37	20 min
	95	5 min
	4	Hold
Second strand synthesis	95	2 min
	4	Hold
	25	5 min
	37	10 min
	70	5 min
	4	Hold
IVT	42	3 h (optional 4 h)
	4	Hold (optional overnight)
	37	15 min
	4	Hold

12. Thoroughly mix and spin down Nuclease Mix (orange). Place on ice.
13. Add 2.0 μl of Nuclease Mix to the sample, and mix thoroughly by flicking the tube, and then spin down.
14. Incubate the sample at 37 °C for 30 min followed by 95 °C for 5 min.
15. Chill the sample to 4–8 °C for at least 1 min. *It is safe to stop at this point in the protocol. You can store the sample overnight at −20 °C.*

Round One: second strand cDNA synthesis

16. Place the sample on 4–8 °C block, and allow to thaw if frozen (at 4–8 °C).
17. Thaw Primer B (Gray-B), mix thoroughly, and spin down.
18. Add 1.0 μl of Primer B. Mix thoroughly by flicking the tube, and spin down.
19. Incubate the sample at 95 °C for 2 min, then chill, and maintain the sample at 4–8 °C for at least 2 min.
20. Thaw Second Strand Master Mix at 4–8 °C (cold block) (Green-1). Mix thoroughly, and spin Second Strand Master Mix.

21. Mix enzyme thoroughly by inverting several times, spin briefly, and then place at 4–8 °C.
22. Add Second Strand Synthesis components separately in the order listed: 29 μl Second Strand Master Mix (Green-1 vial); 1 μl Second Strand Enzyme Mix (Green-2 vial). Total per sample 30 μl.
23. Incubate the sample as follows: 25 °C: 5 min, 37 °C: 10 min, 70 °C: 5 min, 4–8 °C: Hold until ready to proceed (up to a maximum of 30 min).

Round One: cDNA purification

24. Add 250 μl of DNA Binding Buffer (Red-DB) to a DNA/RNA Purification Column seated in the collection tube provided (*see* **Note 17**). Hold for 5 min at room temperature (*see* **Note 18**).
25. Centrifuge at 16,000 × *g* for 1 min.
26. Add 200 μl of DNA Binding Buffer to the Second Strand Synthesis sample tube, mix well, and pipet the entire volume into the purification column.
27. To bind cDNA to the column, centrifuge at 100 × *g* for 2 min (or lowest speed setting available), immediately followed by a centrifugation at 10,000 × *g* for 1 min to remove flow-through.
28. Add 250 μl of DNA Wash Buffer (Red-DW) to the column and centrifuge at 16,000 × *g* for 2 min. Check the purification column for any residual wash buffer. If any wash buffer remains, re-centrifuge at 16,000 × *g* for 1 min.
29. Discard the flow-through and collection tube (*see* **Note 19**).
30. Place the column into the provided 0.5-ml microcentrifuge tube and carefully add 11 μl of DNA Elution Buffer (Red-DE) onto the center of the purification column membrane. (Gently touch the tip of the pipette to the surface of the membrane while dispensing the elution buffer to ensure maximum absorption of DE into the membrane.)
31. Gently tap the purification column to distribute the buffer, if necessary. Incubate for 1 min at room temperature.
32. Place the assembly into the centrifuge as shown, and centrifuge at 1000 × *g* for 1 min, and then centrifuge at 16,000 × *g* for 1 min.
33. Discard the column and retain the elution containing the cDNA in the microcentrifuge tube for further processing (*see* **Note 20**). *It is safe to stop at this point in the protocol. You can store the sample overnight at −20 °C.*

Round One: In Vitro Transcription

34. Thaw IVT Buffer (Blue-1), Master Mix (Blue-2) and Enhancer (Yellow-E) to room temperature and thoroughly mix to dissolve all solids. (IVT Enzyme Mix (Blue-3) does not require thawing and can be put in directly at 4–8 °C.)
35. Mix enzyme thoroughly by inverting several times. Spin briefly.
36. Add IVT components in the order listed: 2 µl IVT Buffer (Blue-1 vial), 6 µl IVT Master Mix (Blue-2 vial), 2 µl IVT Enzyme Mix (Blue-3 vial), 2 µl Enhancer (Yellow-E vial). Total per sample 12 µl. Mix thoroughly by flicking the tube, and then spin down.
37. Incubate at 42 °C for 3 h (Optional: You can use 4-h incubation for additional aRNA yield). Chill the sample(s) to 4–8 °C. *At this point in the protocol, you may hold the reaction mixture at 4–8 °C in the thermal cycler overnight.*
38. Move the samples directly to a 4–8 °C block.
39. Add 1 µl DNase Mix (Blue-4). Mix thoroughly, and spin down.
40. Incubate at 37 °C for 15 min.
41. Chill the sample(s) to 4–8 °C. Proceed immediately to aRNA purification (*see* **Note 21**).

Round One: aRNA Purification

42. Add 250 µl of RNA Binding Buffer (Blue-RB) to a new purification column and incubate for 5 min at room temperature (*see* **Note 22**).
43. Centrifuge at 16,000 × *g* for 1 min.
44. Add 120 µl of RNA Binding Buffer to the IVT reaction sample and mix thoroughly.
45. Pipet the entire volume into the purification column.
46. To bind aRNA, centrifuge at 100 × *g* (or lowest speed setting available) for 2 min, immediately followed by a centrifugation at 10,000 × *g* for 1 min to remove flow-through.
47. Add 200 µl of RNA Wash Buffer (Blue-RW) to the purification column and centrifuge at 10,000 × *g* for 1 min.
48. Add 200 µl of fresh RNA Wash Buffer to the purification column, and centrifuge at 16,000 × *g* for 2 min.
49. Check the purification column for any residual wash buffer. If any wash buffer remains, re-centrifuge at 16,000 × *g* for 1 min.
50. Discard the collection tube and flow-through.
51. Place the purification column into a new 0.5 ml microcentrifuge tube provided in the kit and carefully add RNA Elution Buffer (Blue-RE) directly to the center of the purification

column membrane. Add 30 μl if stopping with one round of amplification or 12 μl if going on to a second round.

52. Gently touch the tip of the pipette to the surface of the membrane while dispensing RE to ensure maximum absorption of RE into the membrane. Gently tap the purification column to distribute the buffer, if necessary.
53. Incubate at room temperature for 1 min.
54. Place each column-tube assembly into the centrifuge rotor with the 0.5 ml tube cap trailing the tube.
55. Centrifuge at 1000 × *g* for 1 min, and immediately centrifuge at 16,000 × *g* for 1 min. Discard the purification column and retain the elution containing the aRNA. *Purified aRNA may be stored at −70 °C. Immediately proceed to Round Two or store the purified aRNA at −70 °C overnight.*

RiboAmp® PLUS Round Two

In second round amplification, purified aRNA product from Round One is used to produce double-stranded cDNA, which in turn is used as a template for an in vitro transcription reaction (*see* **Note 23**). Program thermal cycler as noted in Table 3:

Round Two: first strand cDNA synthesis

56. Thaw samples from round one at 4–8 °C if necessary. Place the samples on a 4–8 °C block.
57. Thaw Primer B (Grey-B), thoroughly mix, spin down, and place on a 4–8 °C block.

Table 3
Thermocycler programming for Arcturus RiboAmp PLUS Round Two

	°C	Time
First strand synthesis	65	5 min
	4	Hold
	25	10 min
	37	45 min
	4	Hold
Second strand synthesis	95	2 min
	4	Hold
	37	15 min
	70	5 min
	4	Hold
IVT	42	4–6 h
	4	Hold (optional overnight)
	37	15 min
	4	Hold

58. Into eluted aRNA product from Round One, add 1.0 μl of Primer B, mix thoroughly by flicking the tube, and spin down.
59. Incubate the microcentrifuge tube at 65 °C for 5 min and then chill the samples to 4–8 °C for 1 min.
60. Spin down the contents and place on 4–8 °C block before proceeding to the next step.
61. Place First Strand Synthesis components (Red-1 and Red-2) at 4–8 °C. First Strand Master Mix must be thawed, thoroughly mixed with all solids dissolved, and maintained at 4–8 °C until used.
62. Add First Strand Synthesis components in the order listed: 7 μl First Strand Master Mix (Red-1 vial), 2 μl First Strand Enzyme Mix (Red-2), total per sample 9 μl Mix thoroughly by flicking the tube, and spin down. *Do not vortex.*
63. Incubate the sample(s) at 25 °C for 10 min and then at 37 °C for 45 min.
64. Chill the sample(s) to 4–8 °C for at least 1 min. *It is safe to stop at this point in the protocol. You can store the sample overnight at −20 °C.*

Round Two: second strand cDNA synthesis

65. Place the sample on a 4–8 °C block and allow it to thaw, if frozen (at 4–8 °C).
66. Thaw Primer A (Gray-A), thoroughly mix, spin down, and place on a 4–8 °C block.
67. Add 1.0 μl of Primer A to the sample. Mix thoroughly by flicking the tube, and spin down.
68. Incubate the sample at 95 °C for 2 min, then cool the sample to 4–8 °C for at least 1 min. Hold the sample at 4–8 °C until ready to proceed.
69. Spin down the contents, and place on 4–8 °C block before proceeding to the next step.
70. Thaw the Second Strand Master Mix (Green-1) at 4–8 °C (cold block). Thoroughly mix and spin Second Strand Master Mix.
71. Add Second Strand Synthesis components separately in the order listed: 29 μl Second Strand Master Mix (Green-1 vial), 1 μl Second Strand Enzyme Mix (Green-2 vial), total per sample 30 μl. Mix thoroughly by flicking the tube, and spin down.
72. Incubate the sample(s) as follows: 37 °C 15 min; 70 °C 5 min; 4–8 °C Hold until ready to proceed (up to a maximum of 30 min).

Round Two: cDNA purification

73. Add 250 µl of DNA Binding Buffer (Red-DB) to a new purification column seated in the collection tube provided. Incubate for 5 min at room temperature (*see* **Note 24**). Centrifuge at 16,000 × *g* for 1 min.
74. Add 200 µl of DNA Binding Buffer to the Second Strand Synthesis sample tube, mix well, and pipette the entire volume into the purification column.
75. To bind cDNA, centrifuge at 100 × *g* (or lowest speed setting available) for 2 min, then immediately centrifuge at 10,000 × *g* for 1 min to remove flow-through.
76. Add 250 µl of DNA Wash Buffer (Red-DW) to the column and centrifuge at 16,000 × *g* for 2 min. Check the purification column for any residual wash buffer. If any wash buffer remains, re-centrifuge at 16,000 × *g* for 1 min.
77. Discard the collection tube and flow-through.
78. Place the column into the provided 0.5-ml microcentrifuge tube and carefully add 11 µl of DNA Elution Buffer (Red-DE) onto the center of the purification column membrane. Gently touch the tip of the pipette to the surface of the membrane while dispensing DE to ensure maximum absorption of DE into the membrane. Gently tap the purification column to distribute the buffer, if necessary.
79. Incubate for 1 min at room temperature.
80. Place each column-tube assembly into the 2 ml support tube in the rotor with the 0.5 ml tube cap trailing the tube.
81. Centrifuge at 1000 × *g* for 1 min, and then immediately centrifuge by 16,000 × *g* for 1 min. Discard the column and retain the elution containing the cDNA. *It is safe to stop at this point in the protocol. You can store the sample overnight at −20 °C.*

Round Two: In vitro transcription

IVT reaction components must be thawed, thoroughly mixed with all solids dissolved, and brought to room temperature just before use.

82. Thaw IVT Buffer (Blue-1), Master Mix (Blue-2) and Enhancer (Yellow-E) to room temperature and thoroughly mix to dissolve all solids.
83. IVT Enzyme Mix (Blue-3) does not require thawing and can be put in directly at 4–8 °C. Mix enzyme thoroughly by inverting several times. Spin briefly.
84. Add IVT components in the order listed: 2 µl IVT Buffer (Blue-1 vial), 6 µl IVT Master Mix (Blue-2 vial), 2 µl IVT

Enzyme Mix (Blue-3 vial), 2 µl Enhancer (Yellow-E vial). Total per sample 12 µl.

85. Incubate at 42 °C for 4 h (*Optional*: If you want additional yield, you can incubate the IVT reaction for up to 6 h). Chill the sample(s) to 4–8 °C. *It is safe to stop at this point in the protocol. You may hold the reaction mixture at 4–8 °C in the thermal cycler overnight.*
86. Move the samples directly to a 4–8 °C block.
87. Add 1 µl DNase Mix (Blue-4). Mix thoroughly and spin down. Incubate at 37 °C for 15 min. Chill the sample(s) to 4–8 °C. Proceed immediately to aRNA purification (*see* **Note 25**).

Round Two: aRNA purification

88. Add 250 µl of RNA Binding Buffer (Blue-RB) to a new purification column seated in the collection tube provided. Incubate for 5 min at room temperature. Centrifuge at 16,000 × *g* for 1 min.
89. Add 120 µl of RNA Binding Buffer to the IVT reaction sample and mix thoroughly. Pipet the entire volume into the purification column.
90. To bind aRNA, centrifuge at 100 × *g* (or lowest speed setting available) for 2 min, and immediately centrifuge at 10,000 × *g* for 1 min.
91. Add 200 µl of RNA Wash Buffer (Blue-RW) to the purification column and centrifuge at 10,000 × *g* for 1 min.
92. Add 200 µl of fresh RNA Wash Buffer to the purification column, and centrifuge at 16,000 × *g* for 2 min. Check the column for any residual wash buffer. If any wash buffer remains, re-centrifuge at 16,000 × *g* for 1 min.
93. Discard the collection tube and flow-through.
94. Place the purification column into a new 0.5 ml microcentrifuge tube provided in the Kit and carefully add 30 µl of RNA Elution Buffer (Blue-RE) directly to the center of the purification column membrane (*see* **Note 26**).
95. Incubate for 1 min at room temperature.
96. Place each column-tube assembly into the 2 ml support tube in the rotor with the 0.5 ml tube cap trailing the tube.
97. Centrifuge at 1000 × *g* for 1 min, followed immediately by 16,000 × *g* for 1 min. Discard the column and retain the elution containing the aRNA.
98. Measure and analyze the aRNA by gel electrophoresis using the Experion StSense Bioanalyzer or Nanodrop (O.D. of the product at A260 and A280). In our experience, both the systems

render similar results after amplification. You can store purified aRNA at −70 °C.

We amplified between 5 and 40 ng of total RNA, obtaining between 5 and 57 μg of mRNA after two rounds.

3.5.2 ExpressArt® TRinucleotide RNA Amplification

The following paragraphs have been mostly elaborated by directly quoting from the manufacturer's protocol:

First Amplification Round

1. Program the thermocycler with temperatures and times according to Table 4.
2. Range of input total RNA for the Nano kit with two amplification rounds: 1–700 ng.

First strand cDNA synthesis

3. Use the first thermocycler program for step ExpArt1 (Table 4).
4. Prepare First Strand cDNA Synthesis Mix 1: 1: 2.4 μl H_2O (Tube 3), 0.8 μl dNTP-Mix (Tube 2), 0.8 μl Primer 1 (Tube 1).

Table 4
Thermocycler programs for ExpressArt First Amplification round

Step	Temperature (°C)	Time	Action
First thermocycler program for step ExpArt1, First Amplification round			
1	65	Hold	Start of first cDNA synthesis. Add 4 μl RNA to 4 μl Mix1
2	65	4 min	
3	37	1 min	
4	37	Hold	Add 8 μl Mix2
5	37	45 min	
6	45	15 min	
7	50	5 min	
8	70	10 min	
9	4	Hold	Put samples on ice and continue
Second thermocycler program for step ExpArt2 for First Amplification round Heating lid is switched off			
1	16	Hold	Add 104 μl Mix1
2	16	1 h	
3	4	Hold	

End of Template DNA synthesis. Spin to collect liquid

5. Add 4 µl Mix 1 to 4 µl of each RNA. Incubate for 4 min at 65 °C in a thermocycler (with heating lid! use standard setting, e.g., 110 °C). Then, cool the samples to 37 °C.
6. In the meantime, prepare the First Strand cDNA Synthesis Mix 2 at room temperature: 3.2 µl DEPC-H_2O (Tube 3), 3.2 µl 5× RT Buffer (Tube 4), 0.8 µl RNase Inhibitor (Tube 5), 0.8 µl. RT Enzyme (Tube 6).
7. Add the First Strand cDNA Synthesis Mix 2 (8 µl) to each sample and mix well. Incubate the samples in a thermocycler: 37 °C/45 min, 45 °C/15 min, 50 °C/5 min, 70 °C/10 min, 4 °C/Hold.
8. Remove the samples from the thermocycler, centrifuge the tubes briefly and put the tubes on ice. Proceed immediately to step ExpArt2.

ExpArt2. Template DNA synthesis

9. Use the second thermocycler program for step ExpArt2. On ice prepare the second strand DNA synthesis mix (Mix 3) in the given order in a 1.5 ml reaction tube: 73.0 µl H_2O (Tube 3), 24.0 µl Polymerase- Buffer (Tube 10), 2.4 µl dNTP-Mix (Tube 2), 3.2 µl Polymerase A (Tube 22), 0.8 µl Polymerase B (Tube 23), 0.8 µl Polymerase C (Tube 24).
10. On ice, add 104 µl of Mix 3 to the first strand reaction. Mix gently by pipetting. Continue incubations: 16 °C/1 h with heating lid switched off. Remove the samples from thermocycler, put on ice. Spin to collect liquid and immediately continue with purification of the Template DNA (step ExpArt3).

ExpArt3. Purification of Template DNA with Spin Columns

11. Before starting, add 16 ml of 100% ethanol to the 4 ml Washing Buffer concentrate (Kit box II) and mix well. Purification Mix 4: 244 µl Binding Buffer (box II), 2 µl Carrier DNA (Tube 14).
12. Add 246 µl of Mix 4 to each Template DNA Reaction (120 µl from step A2). Mix gently by pipetting.
13. Insert DNA Purification Spin Columns in Collection Tubes.
14. Pipette the entire sample onto each column and centrifuge for 1 min at 10,000 × *g* in a table top centrifuge.
15. Discard the flow-through and re-insert the columns in the same Collection Tubes. Add 200 µl Washing Buffer (with Ethanol added) to the columns and centrifuge for 1 min at 10,000 × *g*.
16. Discard the flow-through, re-insert the columns in the same Collection Tubes, and wash again with 200 µl Washing Buffer. Centrifuge for 1 min at 10,000 × *g*.

17. Discard the flow-through, centrifuge again for 1 min at 10,000 × *g*, and discard the Collection Tubes.
18. Insert the columns in fresh 1.5 ml reaction tubes and add 10 μl of Elution Buffer to the columns (make sure to pipette the Elution Buffer exactly in the middle of the column, directly on the top of the matrix, without disturbing the matrix with the pipette tip).
19. Incubate the column for at least 2 min, then centrifuge for 1 min at 10,000 × *g*.
20. The purified template DNA (approximately 8 μl) is now ready for in vitro transcription (step ExpArt4).
21. Alternatively, store the samples at −20 °C for later use.

ExpArt4. Amplification via in vitro Transcription

22. Prepare the in vitro-Transcription Mix 5 by adding the components in the given order: 6.6 μl NTP- Mix (Tube 18), 1.7 μl 10× Buffer (Tube 19), 1.7 μl RNA-Polymerase (Tube 20).
23. Work at room temperature, never on ice, because spermidine in the buffer can cause precipitation of the template DNA.
24. Add 10 μl in vitro-Transcription Mix 5 to template DNA (from A3).
25. Incubate the transcription overnight at 37 °C in a thermocycler with heating lid adjusted to 45 °C; or preferentially in a hybridization oven. Do not use a thermocycler WITHOUT adjustable heating lid, because high lid temperature (usually >100 °C) will negatively affect the efficiency of the transcription reaction!
26. Add 1 μl DNase (Tube 21) to each reaction, mix thoroughly and incubate further at 37 °C for 15 min.
27. Continue with purification of amplified RNA (step ExpArt5).

ExpArt5. aRNA-Purification using RNA spin columns

28. Add 6 ml of 100% ethanol to WB1 (12 ml), and 16 ml of 100% ethanol to WB2 (4 ml), as indicated on the bottles. Put RNase-free water in thermoblock at 95 °C.
29. Add 987 μl of Mix 6 to each in vitro Transcription Reaction. Mix 6: 340 μl RNA LB Buffer (box III), 87 μl RNA DeS Buffer (box III), 560 μl 100% Ethanol (at room temperature; not supplied). Mix thoroughly.
30. Transfer an aliquot of 700 μl of this mixture on the RNA spin column, centrifuge for 30 s at 10,000 × *g* and discard flow-through.
31. Transfer the remaining mixture on column, centrifuge for 30 s at 10,000 × *g*, and discard flow-through.

32. Add 500 µl Wash Buffer 1 (WB 1), centrifuge for 30 s at 10,000 × *g*, and discard flow-through.
33. Add 500 µl Wash Buffer 2 (WB 2), centrifuge for 30 s at 10,000 × *g*, and discard flow-through.
34. Re-insert the column in the same collection tube. Add 500 µl 80% EtOH, centrifuge for 30 s at 10,000 × *g* and discard flow-through.
35. Re-insert the column in the same collection tube, and centrifuge for 1 min at maximum speed to get rid of residual salt on the spin column matrix.
36. Elution: Transfer column in fresh 1.5 ml reaction tube and add 30 µl RNase-free water (preheated at 95 °C).
37. Incubate for 2 min and centrifuge for 1 min at maximum speed. Reapply the eluate on the column. Incubate: 2 min and centrifuge for 1 min at maximum speed.
38. RNA is in a total volume of ~30 µl. Use amplified RNA for second amplification round (section ExpB) or store at −80 °C.

ExpArtB: Second Amplification Round

See **Note 27** for suggestions about RNA analysis previous to the second amplification round.

39. Use thermocycler program for steps ExpArtB1-B2 according to Table 5.
40. Add 5 µl Mix 2-1 to 25 µl of each RNA (for smaller volumes, adjust to with water to a reaction volume of 30 µl). Mix 2-1: 2.5 µl dNTP-Mix Tube 2, 2.5 µl Primer B Tube 8.
41. Incubate for 4 min at 65 °C in a thermocycler (with heating lid! use standard setting, e.g., 110 °C). Cool the samples to 37 °C.
42. In the meantime, prepare the First Strand cDNA Synthesis Mix 2-2 at room temperature (8.4 µl DEPC-H_2O Tube 3, 10.0 µl 5× RT Buffer Tube 4, 0.8 µl RNase Inhibitor Tube 5, 0.8 µl RT). Add the First Strand cDNA Synthesis Mix 2-2 (20 µl) to each sample and mix well by gently flicking the tube.
43. Incubate the samples in a thermocycler: 37 °C/45 min; 45 °C/15 min; 50 °C/5 min; 37 °C/Hold.
44. Then add 5 µl Primer Erase Mix 2-3 (3 µl DEPC-H_2O Tube 3; 1 µl 5× Extender Buffer Tube 9; 1 µl Primer Erase Tube 11), and continue incubations: 37 °C/5 min, 80 °C/15 min, 37 °C/Hold.
45. Add 5 µl of RNase Mix 2-4 to First Strand cDNA Reaction (3 µl DEPC-H_2O Tube 3; 1 µl 5× Extender Buffer Tube 9; 1 µl RNase Tube 7). Incubate for 20 min at 37 °C.

Table 5
Thermocycler program for steps ExpArtB1 /B2 for second Amplification round

Step	Temperature (°C)	Time	Action
1	65	Hold	Start of first cDNA synthesis. Add 25 μl RNA-a1 to 5 μl Mix 2-1
2	65	4 min	
3	37	1 min	
4	37	Hold	Add 20 μl Mix 2-2
5	37	45 min	
6	45	15 min	
7	50	5 min	
8	37	1 min	
9	37	Hold	Add 5 μl Mix 2-3
10	37	5 min	
11	80	15 min	
12	37	1 min	
13	37	Hold	Add 5 μl Mix 2-4
14	37	20 min	
15	37	Hold	Add 35 μl Mix 2-5
16	96	1 min	
17	37	1 min	
18	37	Hold	Add 20 μl Mix 2-6
19	37	30 min	
20	65	15 min	
21	4	Hold	

End of Template DNA synthesis. Spin to collect liquid

ExpArtB2. Template DNA synthesis

46. Add 35 μl of Mix 2-5 to each First Strand cDNA Synthesis Reaction. Second Strand cDNA Synthesis Mix 2-5: 10.0 μl DEPC-H_2O Tube 3; 10.0 μl 5× Extender Buffer Tube 9; 12.5 μl Primer C Tube 12; 2.5 μl dNTP-Mix Tube 2.
47. Incubate as follows in a thermocycler: 96 °C/1 min, 37 °C/ 1 min.
48. Add 20 μl of Extender Enzyme B Mix 2-6 (18 μl DEPC-H_2O Tube 3, 1 μl 5× Extender Buffer Tube 9, 1 μl Extender

Enzyme B Tube 13) to each sample and mix well by gently flicking the tube.

49. Continue the incubation: 37 °C/30 min, 65 °C/15 min, 4 °C/Hold. After incubation, place the tubes on ice. Proceed to step ExpArtB3.

ExpArtB3. Purification of Template DNA with Spin Columns

50. Add 236 µl of Mix 2-7 (234 µl Binding Buffer (box II), 2 µl Carrier DNA Tube 14) to each Template DNA Reaction (115 µl from step B2). Mix gently by pipetting.
51. Insert DNA Purification Spin Columns in Collection Tubes.
52. Pipette the entire sample onto each column and centrifuge for 1 min at 10,000 × *g* in a table top centrifuge (*see* **Note 28**).
53. Discard the flow-through and re-insert the columns in the same Collection Tubes. Add 200 µl Washing Buffer (with Ethanol added) to the columns and centrifuge for 1 min at 10,000 × *g*.
54. Discard the flow-through, re-insert the columns in the same Collection Tubes, and wash again with 200 µl Washing Buffer. Centrifuge for 1 min at 10,000 × *g*.
55. Discard the flow-through, centrifuge again for 1 min at 10,000 × *g*, and discard the Collection Tubes.
56. Insert the columns in fresh 1.5 ml reaction tubes and add 10 µl of Elution Buffer to the columns (*see* **Note 29**).
57. Incubate the column for at least 2 min, then centrifuge for 1 min at 10,000 × *g*. The purified template DNA (approximately 8 µl) is now ready for in vitro transcription. Alternatively, store the samples at −20 °C for later use.

ExpArtB4. Amplification via in vitro Transcription

58. Prepare the in vitro-Transcription Mix 5 by adding the components in the given order: 6.6 µl NTP-Mix Tube 18, 1.7 µl 10× Buffer Tube 19, 1.7 µl T7 RNA Polymerase Tube 20. Work at room temperature (when cold spermidine in the buffer can cause precipitation of DNA template).
59. Add 10 µl in vitro-Transcription Mix 5 to template DNA (from B3).
60. Incubate the transcription overnight at 37 °C in a thermocycler with heating lid adjusted to 45 °C; or preferentially in a hybridization oven (*see* **Note 30**).
61. Add 6 ml of 100% ethanol to WB1 (12 ml), and 16 ml of 100% ethanol to WB2 (4 ml), as indicated on the bottles. At the start, put RNase-free water in thermoblock at 95 °C.

62. Add 987 μl of Mix 6 (340 μl RNA LB Buffer (box III), 87 μl RNA DeS Buffer (box III), 560 μl 100% Ethanol at room temperature; not supplied) to each in vitro Transcription Reaction. Mix thoroughly.
63. Transfer an aliquot of 700 μl of this mixture on the RNA spin column, centrifuge for 30 s at 10,000 × *g*, and discard flow-through.
64. Transfer the remaining mixture on column, centrifuge for 30 s at 10,000 × *g*, and discard flow-through.
65. Add 500 μl Wash Buffer 1 (WB 1), centrifuge for 30 s at 10,000 × *g*, and discard flow-through.
66. Add 500 μl Wash Buffer 2 (WB 2), centrifuge for 30 s at 10,000 × *g*, and discard flow-through.
67. Re-insert the column in the same collection tube. Add 500 μl 80% EtOH, centrifuge for 30 s at 10,000 × *g*, and discard flow-through.
68. Re-insert the column in the same collection tube, and centrifuge for 1 min at maximum speed to get rid of residual salt on the spin column matrix.
69. Elution: Transfer column in fresh 1.5 ml reaction tube and add 30 μl RNase-free water (preheated at 95 °C).
70. Incubate for 2 min and centrifuge for 1 min at maximum speed. Reapply the eluate on the column.
71. Incubate for 2 min and centrifuge for 1 min at maximum speed. RNA is in a total volume of ~30 μl. Use amplified RNA immediately or store at −80 °C.
72. In our experiments, mRNA concentration and purity was assessed again both by electrophoresis (BioRad ExperionSt-Sens kit) and by spectrophotometry (Nanodrop). We amplified 3.7–37 ng of total RNA, obtaining between 6 and 21 μg of mRNA after two rounds. (*See* Table 6 for expected yields of amplified RNA according to the manufacturer.)

3.6 Retrotranscription and Taqman Low Density Arrays

3.6.1 Retrotranscription of ExpressArt Amplified RNA

1. ExpressArt amplified RNA was reversely transcribed with ExpressArt® TR cDNA synthesis kit (no. 8994-A30, AmpTec, AMSBIO, UK).
2. Preparation of amplified RNA stock: we calculate the amount of each sample that includes 500 ng of amplified RNA (0.5–3 μl, depending on the sample), and dilute them to 10 μl, to obtain a 50 ng/μl stock. From this stock we will use 4 μl (200 ng) per RT reaction. Manufacturer's instructions are quoted:

Table 6
Expected yields of amplified RNA according to the manufacturer

Input total RNA	RNA-a1 first round	RNA-a2 second round
200 ng	4 ± 2 μg	With 200 ng RNA-a1: 50 ± 20 μg
100 ng	2 ± 1 μg	With 200 ng RNA-a1: 50 ± 20 μg
50 ng	1 ± 0.3 μg	With 200 ng RNA-a1: 50 ± 20 μg
10 ng	200 ± 50 ng	Using all of RNA-a1: 50 ± 20 μg
≤1 ng	20 ± 10 ng	Using all of RNA-a1: 20 ± 10 μg

3. *First strand cDNA synthesis*: Prepare First Strand cDNA Synthesis Mix 1: 2.4 μl DEPC-H_2O (Tube 3), 0.8 μl dNTP-Mix 10 mM (Tube 2), 0.8 μl Primer TR (Tube 1).
4. Add 4 μl Mix 1 to 4 μl of RNA stock solution (50 ng/μl). Incubate for 4 min at 65 °C in a thermocycler (with heating lid at standard setting, e.g., 110 °C).
5. Cool the samples to 37 °C.
6. In the meantime, prepare the First Strand cDNA Synthesis Mix 2 at room temperature: 4.2 μl DEPC-H_2O (Tube 3), 3.2 μl 5× RT Buffer (Tube 4), 0.3 μl RNase Inhibitor (Tube 5), 0.3 μl RT Enzyme (Tube 6).
7. Add the First Strand cDNA Synthesis Mix 2 (8 μl) to each sample and mix well by gently flicking the tube.
8. Incubate the samples in a thermocycler: 37 °C/45 min; 45 °C/15 min; 50 °C/5 min; 70 °C/10 min; 4 °C/Hold.
9. Spin briefly to collect liquid.
10. *Purification of single-stranded cDNA with Spin Columns*: Before starting, add 15 ml of 100% ethanol to the 3 ml Wash Solution concentrate (Kit box II) and mix well.
11. Add 284 μl of Mix 3 (250 μl Binding Solution-BS, 34 μl DEPC-H_2O -Tube 3-) to each cDNA Reaction (16 μl). Mix gently by pipetting.
12. Insert DNA Purification Spin Columns in Collection Tubes, pipette the entire sample onto each column, and centrifuge for 1 min at 10,000 × *g* in a table top centrifuge.
13. Discard the flow-through and re-insert the columns in the same Collection Tubes. Add 200 μl Wash Solution WS (with

Ethanol added) to the columns and centrifuge for 1 min at 10,000 × *g*.

14. Discard the flow-through, re-insert the columns in the same Collection Tubes, and wash again with 200 µl Wash Solution WS. Centrifuge for 1 min at 10,000 × *g*.
15. Discard the flow-through and the Collection Tubes. Insert the columns in fresh 1.5 ml reaction tubes and add 25 µl of Elution Buffer to the columns (*see* **Note 29**). Incubate the column for at least 2 min, then centrifuge for 1 min at 10,000 × *g*.
16. Repeat the elution step with a second aliquot of 25 µl Elution Buffer.
17. The purified template DNA (approximately 48 µl) is now ready for qPCR analysis or, alternatively, stored at −20 °C for later use.
18. We dilute cDNA to 50 µl (with 2 µl water). This cDNA comes from retrotranscription of 200 ng of amplified RNA (*see* **Note 31**).

3.6.2 Retrotranscription of RiboAmp Amplified RNA

1. RiboAmp amplified RNA was reversely transcribed with a High Capacity RNA to cDNA kit (Applied Biosystems, USA). Manufacturer's instructions are quoted:
2. Input Amount of Total RNA: Up to 2 µg of total RNA per 20-µl reaction (We used 1 µg of amplified RNA).
3. Allow the kit components to thaw on ice and prepare the RT reaction mix on ice using the kit components before preparing the reaction plate (per 20-µl reaction): 10 µl 2× RT Buffer Mix, 1.0 µl 20× RT Enzyme Mix, up to 9 µl RNA sample, Nuclease-free H_2O (quantity sufficient to 20 µl) (*see* **Note 32**).
4. Mix gently and place the reaction mix on ice.
5. Prepare the cDNA Reverse Transcription Reactions: Aliquot 20 µl of RT reaction mix into each well, or tube; seal the plates or tubes. Briefly centrifuge the plate or tubes to spin down the contents and to eliminate air bubbles.
6. Place the plate or tubes on ice until you are ready to load the thermal cycler.
7. Program the thermal cycler conditions: 37 °C/60 min, 95 °C/5 min, 4 °C/Hold.
8. Set the reaction volume to 20 µl.
9. Load the reactions into the thermal cycler and start the reverse transcription run.
10. You can store cDNA RT tubes for short-term (up to 24 h before use) at 2–8 °C; for long term, store them at −15 to −25 °C.

3.6.3 Taqman Low Density Arrays (TLDAs)

1. To test gene expression profiles we designed custom Taqman Low Density Arrays in which we loaded pre-designed and validated Applied Biosystems™ TaqMan® Gene Expression Assays for either rats or humans as required. Those assays included endocannabinoid system components genes [13], Wnt family members genes [10], and neurogenic or ependymoma-related genes [11]. Based on our preliminary data and manufacturer's technical recommendations, we chose 18S gene as an endogenous control [10, 11].
2. For qPCR single assays, we normally dilute cDNA to 100 μl (1:2 dilution) and use 1.25–2.5 μl of cDNA per assay (depending on the gene abundance). For a reference, the amount of 1.25 μl is approximately equivalent to 1.25 ng of ExpressArt amplified RNA or 5–25 ng of starting total RNA.
3. For TLDAs, we used between 60- or 120 ng of cDNA (*see* **Note 33**) premixed with TaqMan® Universal Master Mix II which were loaded into each loading port of TLDAs (each port delivers to 48 single wells, meaning that we use 1.25–2.5 ng cDNA/well) and cards were centrifuged and run on an Applied Biosystems® 7900HT Fast Real-Time PCR System.
4. Automatic detection of Ct was used to establish the threshold of amplification for each gene.
5. We used Cts average from technical duplicates, and calculated ΔCt, the difference between the Ct of each gene and the Ct of the endogenous gene (18S) for that sample.
6. Only genes consistently expressed in the ependymal region were studied. Consistent expression was defined as expression in at least three out of five (rats) or four (human) samples.
7. With those genes, we performed statistical comparisons to determine enrichment due to age (young vs mature), species (rats vs humans), or regions (ependymal region vs ventral horn).
8. Statistical comparisons can be performed with ΔCts using Student's *t*-test with multiple testing correction by Benjamini-Hochberg using R Stats package [16, 17].
9. Folds of enrichment between groups can be detected using Relative quantity (RQ), defined as $2^{\Delta\Delta Ct}$, where ΔΔCt for each gene is the difference between the average ΔCt in the group 1 (species1, age1, region1) minus the average value of expression in group 2 (species2, age2, region2).

4 Notes

1. DEPC precautions: If trace amounts of DEPC are not removed, they can modify purine residues in the RNA by carboxymethylation, which impairs in vitro translation systems. DEPC is toxic and suspected carcinogen and must be handled with caution. Do not use DEPC to treat Tris solutions since it is inactivated.
2. According to Leica, UV treatment not only may destruct RNases but also reduce electrostatical charge of the foil.
3. A very useful and convenient alternative is to use a wire side cutter with slightly bended tips. They can be found in any hardware store and allow for a safe and quick bone cutting.
4. Excess of embedding compound can interfere with cutting during LCMD. In the case that samples are already surrounded by OCT, trim it away as much as possible. If the tissue is to be immediately sectioned, then slide mounted sections can be washed in 70% ethanol to clear remaining OCT.
5. Since microscopes may not reach the whole range of the PEN membrane, avoid using the external edges of the slide membrane, leaving 4–5 mm free of tissue from the edge of the slide.
6. We have always used tissue sectioned immediately before staining and LCMD. In some manuals it is also suggested that the dried sections can be stored in tightly closed bags, slide boxes, or other containers containing desiccant at −80 °C for several months. For us and others [18], it is best to store the block of tissue. Anyhow, it is crucial to avoid formation of water condensation inside the container both for RNA quality, and for a proper drop of the tissue after LCMD.
7. We recommend processing one slide at a time and reducing the duration to approximately 60–90′ from the tissue section to collection in the extraction buffer. We collect samples from different slides, usually 3, in the same eppendorf by coordinating the work of two persons: right after toluidine blue staining, one person starts again the process of collecting tissue on a second PEN-slide, while the other one takes care of the Laser Microdissection. This makes the second slide ready immediately after the first one has been completely microdissected.
8. Do not re-draw line with laser more than twice. It greatly reduces RNA quantity and integrity.
9. Spectrophotometric measurements are not reliable below 1 μg of RNA.

10. If an aliquot of denatured, diluted RNA ladder exists, thaw it on ice and proceed to **step 5**. DO NOT reheat diluted RNA ladder.
11. This is a critical point. For avoiding bubbles, it is advised to pipette volumes slow but continuously, close to the bottom of each well. Anyway, if bubbles are formed, they can be pricked with a fine needle without touching the bottom of the well. When priming is properly done, it should be hard to distinguish microchannels. If visual inspection after priming finds bubbles or partially incomplete priming, discard the chip.
12. Total RNA assay estimates both quantity, purity and RQI score, whereas mRNA measurements will not render 18S/28S ratio neither RQI estimation.
13. Both quantification and the integrity score calculations are based on the ladder separation. If ladder does not run properly, chip should be repeated.
14. It should be taken into account that the strategy used for RNA amplification used in the ExpressArt® TRinucleotide mRNA Amplification Kit produces aRNA and further cDNA able to be used with PCR and genomic arrays but not with RNASeq techniques (checked with AMSBIO technical service).
15. Overloading high sensitivity kits may deplete key components during the reaction and may lead to no amplification. Choose the appropriate kit (RiboAmp® PLUS or RiboAmp® HS PLUS) according to the total RNA input requirements (Minimum input will yield >15 μg of aRNA, recommended input will yield >30 μg of aRNA):

    RiboAmp® PLUS 2-round: Minimum input 5 ng, recommended input 10 ng–40 ng.
    RiboAmp® HS PLUS 2-round: Minimum input 100 pg, recommended input 500 pg-5 ng.
16. Use the following tips for the amplification protocol: (a) When adding reagent to samples or master mixes, pipette mixtures up and down several times to ensure complete transfer of reagent from the pipette tip. (b) Prior to the first use of an enzyme, gently mix (do not vortex) and briefly microcentrifuge the vial to ensure that all enzyme is mixed and collected at the bottom of the vial. (c) Keep thawed reagents and reaction tubes in cold blocks at 4–8 °C while adding reagents to samples. (d) Prior to each incubation, mix the samples thoroughly by flicking the reaction tube (unless noted in protocol) to ensure optimal performance. Spin down before proceeding. *Do not vortex.* (e) Use a microcentrifuge to spin down all components and samples following each mixing step.

17. DNA Binding Buffer (Red-DB) must be at room temperature and mixed thoroughly by shaking before use. A precipitate may form during long-term storage. Dissolve precipitate prior to use by mixing. If necessary, warm the DB vial to redissolve.
18. Improper orientation of tubes during centrifugation may result in cap breakage or sample loss. To use the column-tube assembly correctly: (1) Insert a spin column into the 0.5 ml tube, aligning the two cap hinges; (2) Load Elution Buffer onto the column and incubate as directed; (3) Place the column-tube assembly into a 2 ml lidless support tube in the centrifuge rotor.
19. IMPORTANT! Avoid splashing flow-through in the collection tube onto the column. If flow-through waste liquid wets the outside of the purification column, re-centrifuge the column at 16,000 $\times g$ to remove liquid.
20. IMPORTANT! To avoid potential breakage of the microcentrifuge tube cap during centrifugation, insert the purification tube/0.5 ml tube assembly into a lidless 1.7/2.0 ml tube. Insert this assembly into adjacent rotor holes as illustrated. Rest the tube cap against the tube immediately clockwise to it. Place an empty, lidless 1.7/2.0-ml tube into the rotor hole adjacent in the clockwise direction to the last assembly.
21. RNA may be adversely affected if not purified immediately after DNase treatment.
22. RNA Binding Buffer (Blue-RB) must be at room temperature and thoroughly mixed before use. A precipitate may form during long-term storage. Dissolve precipitate prior to use by mixing. If necessary, warm the RB vial to redissolve.
23. There are two significant differences between the first-round and second-round amplification protocols: (1) Since Primer A is a component of Second Strand Synthesis, and Primer B is a component of First Strand Synthesis, reaction temperatures and incubation intervals are different (2). The second-round amplification protocol does not make use of First Strand Nuclease Mix. The aRNA product produced after the second round of amplification is somewhat shorter than that formed from one round. Typically, the bulk of the aRNA visualized through gel electrophoresis will range from under 200 to over 600 bases.
24. RNA Binding Buffer (Red-RB) must be at room temperature and thoroughly mixed before use. A precipitate may form during long-term storage. Dissolve precipitate prior to use by mixing. If necessary, warm the RB vial to redissolve.

25. DNase Mix must be thoroughly mixed and held at 4 °C until used. RNA may be adversely affected if not purified immediately after DNase.
26. Gently touch the surface of the membrane with the tip of the pipette while dispensing RE to ensure maximum absorption of RE into the membrane. Gently tap the purification column to distribute the buffer, if necessary.
27. If input RNA amounts below 50 ng were used, the maximum volume of 25 μl aRNA is used for the second amplification round. Important when using photometric quantification (Nanodrop): The in vitro transcription reactions are performed with very high NTP concentrations (30 mM total) and these NTP's are not removed 100% by RNA cleanup with spin columns. In consequence: measurements with negative control reactions may indicate—erroneous—values of up to approximately 1.5 μg. The presence of RNA strongly competes and prevents this "background binding" of NTP's, and this means, calculated yields of >2 μg are a reliable indication of RNA amounts. A correlation with electrophoretic results (BioRad Experion or agarose gel) is recommended. If >50 ng of input total RNA were used, the expected yields of amplified RNA are >2 μg. If an additional second amplification round (*see* section B) is required, a maximum 0.8 μg of amplified RNA can be used—RNA yields should be confirmed by electrophoresis. Quality Control with ExpressArt: All ionic compounds interfere with capillary electrophoresis.

    Amplified RNA is again reverse transcribed into cDNA to produce high yields of aRNA via a second round of amplification. Manufacturer recommends using approximately 200 ng amplified RNA from the first amplification round (a maximum volume of 25 μl of aRNA from step ExpArt5). Please do not use more than 1 μg of amplified RNA.
28. Guanidine thiocyanate in the Binding Buffer is an irritant. Always wear gloves and follow standard safety precautions to minimize contact when handling.
29. Make sure to pipette the Elution Buffer exactly in the middle of the column, directly on the top of the matrix, without disturbing the matrix with the pipette tip.
30. Do not use a thermocycler WITHOUT adjustable heating lid, because high lid temperature (usually >100 °C) will negatively affect the efficiency of the transcription reaction!
31. Some assays offer an estimation of how much RNA load in TLDAs based on "total RNA" estimations from a non-amplified sample (that includes all types of RNA). Since we will use samples already enriched in mRNA after two-rounds of amplification, we need some equivalence to estimate the amount of cDNA to be loaded. In our preliminary

tests, we estimated that 100–120 ng of aRNA/cDNA may roughly approximate to the suggested 500 ng of total RNA in terms of loading TLDAs.

32. Include additional reactions in the calculations to provide excess volume for the loss that occurs during reagent transfers.
33. With the stocks obtained from amplification, the use of more than 30–40 μl of cDNA stock (120 ng aRNA-cDNA) is not well tolerated by TLDAs and may cause problems with salt concentrations rendering inefficient PCR reactions.

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Chapter 18

Isolation of Amyloid Plaques and Neurofibrillary Tangles from Archived Alzheimer's Disease Tissue Using Laser-Capture Microdissection for Downstream Proteomics

Eleanor Drummond, Shruti Nayak, Geoffrey Pires, Beatrix Ueberheide, and Thomas Wisniewski

Abstract

Here, we describe a new method that allows localized proteomics of amyloid plaques and neurofibrillary tangles (NFTs), which are the two pathological hallmarks of Alzheimer's disease (AD). Amyloid plaques and NFTs are visualized using immunohistochemistry and microdissected from archived, formalin-fixed paraffin-embedded (FFPE) human tissue samples using laser-capture microdissection. The majority of human tissue specimens are FFPE; hence the use of this type of tissue is a particular advantage of this technique. Microdissected tissue samples are solubilized with formic acid and deparaffinized, reduced, alkylated, proteolytically digested, and desalted. The resulting protein content of plaques and NFTs is determined using label-free quantitative LC-MS. This results in the unbiased and simultaneous quantification of ~900 proteins in plaques and ~500 proteins in NFTs. This approach permits downstream pathway and network analysis, hence providing a comprehensive overview of pathological protein accumulation found in neuropathological features in AD.

Key words Amyloid plaques, Neurofibrillary tangles, Laser-capture microdissection, Mass spectrometry, Formalin-fixed paraffin embedded, Alzheimer's disease

1 Introduction

Amyloid plaques and neurofibrillary tangles (NFTs) are the two pathological hallmarks that define Alzheimer's disease (AD). While plaques and NFTs primarily consist of aggregated beta amyloid (Aβ) and tau respectively, targeted immunohistochemistry studies have shown that they also contain a diverse range of other proteins. Amyloid plaques contain many amyloid binding proteins (such as apolipoprotein E, clusterin, ubiquitin, etc.) and proteins found in astrocytes, microglia, and dystrophic neurites that surround and infiltrate plaques. NFTs contain phosphorylated tau binding proteins, such as heat shock proteins, cytoskeletal proteins, and kinases [1]. The presence and abundance of proteins besides Aβ in plaques

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and tau in NFTs may have an important role in the development or spread of these neuropathological features during disease. However, the methodology available to comprehensively characterize and quantify these proteins has been limited; restricted to targeted immunohistochemistry studies or co-immunoprecipitation biochemical studies. Both of these techniques rely on targeted analysis and hence make discovery of novel plaque and NFT-associated proteins impossible.

Therefore, we have developed a localized proteomics method that combines laser-capture microdissection (LCM) and label-free quantitative mass spectrometry (LC-MS). This strategy allows the detection and quantification of plaque and NFT-associated proteins isolated from archived human brain tissue. The vast majority of human brain tissue specimens available for research are formalin-fixed, paraffin-embedded (FFPE) blocks of tissue collected at autopsy. Traditionally, this type of tissue has been neglected in proteomic research studies because it was thought to be only suitable for immunohistochemistry or histology. Researchers have avoided using FFPE tissue for proteomics in the past because of concerns that the protein crosslinks generated during FFPE processing will prevent the detection of proteins using mass spectrometry or lead to inferior results compared to those using frozen, unfixed tissue. However, we (and an increasing number of other researchers, primarily in the cancer field) have shown that this is not the case [2–6]. The method described below has been specifically optimized to use FFPE tissue because the abundance of human FFPE tissue specimens is a potential gold mine for future studies of human disease. A limited number of previous studies have performed proteomics on plaques and NFTs microdissected from human brain samples; however, these have all used frozen tissue [7–11]. The method we describe below is a significant improvement over the ones described previously, because it results in a greater number of proteins detected. We have also determined that an initial solubilization step with formic acid is essential for detection of multiple proteins present in plaques and NFTs that are of particular interest in AD (most notably Aβ and tau), while not affecting the detection of abundant soluble proteins [2].

Another important aspect of the method described below is the use of LCM to isolate plaques and NFTs from human brain sections. Successful isolation of neuropathological features from human brain samples has been a technical roadblock that has prevented the comprehensive analysis of proteins specifically localized in plaques and NFTs. LCM bypasses this complication; using a precise laser to dissect microscopic regions of interest without affecting the protein content of the isolated tissue, therefore generating a tissue sample that contains the highest purity possible of

plaques and NFTs. The time-consuming nature of LCM is one of the concerns with using this technique; therefore in our previous studies we optimized the minimal amount of tissue required for downstream LC-MS. We have shown that 1.5 mm^2 total tissue area microdissected from 8 μm thick tissue sections is adequate for consistent LC-MS analysis [2]. It takes approximately 1.5 h to microdissect 1.5 mm^2 total tissue area of plaques and approximately 8 h to microdissect 1.5 mm^2 total tissue area of NFTs.

Analysis of the protein content of plaques and NFTs is performed using LC-MS. Previous studies have relied on targeted, semi-quantitative techniques such as immunohistochemistry, Western blot, or ELISA for protein detection. Unbiased, quantitative mass spectrometry has many benefits over these traditional techniques; (a) it does not rely on the availability, sensitivity, and specificity of antibodies, (b) it is untargeted and therefore allows the unbiased identification of novel proteins in AD pathogenesis, (c) it allows the concurrent identification of hundreds of proteins at once, which then allows for pathway/network analysis of AD pathogenesis, giving a much richer picture of the molecular mechanisms that underlie AD.

We have optimized the mass spectrometry conditions described below to permit maximum protein identification using the minimal amount of microdissected tissue. The dynamic range of mass spectrometry is below the dynamic range of protein concentration found in most systems; therefore, the entire proteome cannot be detected by a single mass spectrometry experiment. Generally, fractionation or enrichment strategies are needed to increase the proteome coverage and allow comprehensive detection of the protein composition of a given system. Here, the use of LCM to isolate defined pathological features (plaques or NFTs) allows us to focus our analysis and thus greatly reduces the proteome complexity, enabling reasonable proteome coverage without the need of extensive fractionation. The microscopic amounts of tissue used for this technique means that a label-free quantitative strategy rather than multiplexed quantitation using isobaric tags is recommended. In our hands labeling with multiplexing reagents leads to less proteins being detected as the extra sample manipulation during labeling with the multiplexing reagents increases the sample loss. Furthermore, the microscopic amount of starting material makes the minimization of contamination and choice of tube for the collection of LCM dissected tissue critical (*see* Subheading 4). Tubes should be carefully tested in preliminary studies to ensure that they do exacerbate sample loss. We also recommend use of a sensitive mass spectrophotometer that offers high resolution and mass accuracy (<10 ppm).

2 Materials

2.1 Tissue Sectioning, Staining, and LCM

1. Microtome (Leica RM2255).
2. Fisher Tissue Flotation Bath.
3. FFPE archived tissue blocks.
4. PET membrane FrameSlides (Leica).
5. Xylenes (Histological Grade).
6. Ethanol (100%).
7. Staining containers (we use typical glass staining containers with a corresponding glass slide holder, but any staining containers suitable for histological staining will work).
8. Slide box (for drying and storing slides).
9. Laser-capture microdissection microscope (we use a Leica LMD6500).
10. Collection tubes (Axygen™ PCR tubes with 0.5 ml flat cap).
11. Phosphate-buffered saline (PBS).
12. ImmEdge Hydrophobic Barrier PAP Pen (Vector).
13. Normal goat serum (NGS; MP Biomedicals).
14. Hydrogen peroxide (H_2O_2; Fisher H323-500).
15. VECTASTAIN Elite ABC HRP Kit (Vector).
16. Metal enhanced DAB substrate Kit (Thermo Scientific).
17. 4G8 anti-Aβ primary antibody (BioLegend).
18. AT8 anti-phosphorylated tau primary antibody (Thermo Scientific).
19. Alex Fluor 488-conjugated goat anti-mouse IgG, Fcγ fragment specific (Jackson ImmunoResearch).
20. Biotinylated horse anti-mouse IgG antibody (Vector).

2.2 LC-MS

1. Water, LC-MS grade.
2. Acetic acid, mass spectrometry grade.
3. Formic Acid LCMS grade (Thermo Scientific).
4. Trifluoroacetic Acid (TFA), sequencing grade (Thermo Scientific).
5. Ammonium bicarbonate.
6. Acetonitrile, LC-MS grade (Fisher).
7. Methanol, LC-MS grade (Fisher).
8. Ethanol, LC-MS grade (Acros).
9. Bench top centrifuge (Eppendorf).
10. Thermomixer R (Eppendorf).

11. Dithiothreitol (DTT).
12. Iodoacetamide (IA).
13. Sequencing grade trypsin (Promega).
14. Speed Vacc Concentrator.
15. Ultrasonic cleaner.
16. pH indicator strips pH 0–14.
17. POROS 50 R2 (Applied Biosystems). Weigh out 500 mg of POROS R2 50 μm and place into a 15 ml falcon tube. Wash it with 10 ml of Optima LC/MS methanol. Vortex gently until all of the beads are in methanol. Let the beads settle to the bottom of the tube. Discard the methanol and wash with 10 ml of Optima LC/MS acetonitrile, followed by 95% acetonitrile in 0.1% TFA as above. Wash with 10 ml of Optima LC/MS water. Repeat the water wash step two more times. Resuspend the POROS beads in 10 ml of 10% ethanol (final concentration 50 mg/ml). Wrap the lid with parafilm and store at 4 °˜C. This POROS bead slurry can be stored at 4 °˜C for about 2–-3 months. Alternatively, C18 ZipTips (see below) or StageTips [12] can be used instead of the POROS beads.
18. C18 ZipTip (Millipore).
19. Autosampler vial (Fisher Scientific).
20. Autosampler vial caps (Fisher Scientific).

We used the following LC-MS setup for our studies; however, this can be replaced with other nano-LC columns and high-resolution mass spectrometers.

21. Easy spray PepMap column 75 μm × 50 cm (Thermo Scientific).
22. Acclaim PepMap 100 pre-column (Thermo Scientific).
23. Easy nLC 1000 HPLC system (Thermo Scientific).
24. QExactive Mass Spectrometer (Thermo Scientific).

3 Methods

3.1 FFPE Tissue Sectioning

1. Clean microtome and water bath with picopure water and RNAse Away (Thermo Fisher) to minimize likelihood of contamination. *See* **Note 1**
2. Cut 8 μm thick sections from FFPE tissue blocks using a microtome. *See* **Note 2**
3. Place tissue sections onto the surface of a water bath.
4. Collect sections onto LCM-compatible PET slides.

5. Leave the slides to dry in a closed container (e.g., slide box) for at least 24 h to ensure that all residual water from the sectioning process has evaporated prior to staining (*see* **Note 3**).

3.2 Fluorescent Immunohistochemistry (Plaques)

1. Dewax sections in xylene (2 × 30 s), followed by a series of graded ethanol washes (100% ethanol (2 × 30 s), 95% ethanol (1 min), 70% ethanol (1 min). *See* **Note 4**
2. Wash with PBS (3 × 5 min).
3. Outline sections with PAP Pen.
4. Wash with PBS (2 × 5 min). *See* **Note 5**
5. Block sections for 1 h at room temperature (RT) in 10% NGS diluted in PBS.
6. Incubate the slides overnight at 4 °C with 4G8 anti-Aβ primary antibody (1:1000 in 4% NGS diluted in PBS).
7. Wash the slides with PBS (3 × 5 min).
8. Incubate the sections with 488 fluorescent secondary antibody (1:500 in PBS) for 2 h at RT (*see* **Note 6**).
9. Wash with PBS (3 × 5 min).
10. Rinse with double distilled MilliQ water to remove residual salt from staining procedure.
11. Allow the slides to dry in a closed container (e.g., slide box) to avoid contamination with dust.

3.3 Peroxidase Immunohistochemistry (Neurofibrillary Tangles) (See Note 7)

1. Dewax sections in xylene (2 × 30 s), followed by a series of graded ethanol washes (100% ethanol (2 × 30 s), 95% ethanol (1 min), 70% ethanol (1 min))
2. Wash with PBS (3 × 5 min).
3. Outline sections with PAP Pen.
4. Incubate the slides with 0.3% H_2O_2 in PBS for 20 min.
5. Wash the slides PBS (3 × 5 min).
6. Block sections for 1 h at RT with 10% NGS diluted in PBS.
7. Incubate the slides overnight at 4 °C with AT8 primary antibody (1:500 in 4% NGS diluted in PBS).
8. Wash the slides with PBS (3 × 5 min).
9. Incubate sections with biotinylated anti-mouse IgG (1:1000 in PBS) for 2 h at RT.
10. 30 min prior to the end of incubation, make ABC solution by mixing 1 drop of solution A and 1 drop of solution B in 10 mL PBS. Slowly mix solution using a rotator until use.
11. Wash the slides with PBS (3 × 5 min).
12. Incubate the slides with ABC solution for 1 h at RT.
13. Wash the slides with PBS (3 × 5 min).

14. Incubate the slides with DAB solution (diluted 1:10 in a peroxidase solution provided in the kit) until appropriate color development. *See* **Note 8**.
15. Quickly rinse the slides with PBS.
16. Wash the slides with PBS (2 × 5 min) using a coplin jar.
17. Quickly rinse sections with ddH_2O.
18. Leave the slides to dry in a closed slide box to avoid contamination.

3.4 Laser-Capture Microdissection

1. Load the slides onto an LCM microscope with the tissue facing downward toward the collector (*see* **Note 9**).
2. Load two 0.5 ml collection tubes into the collector; the first tube containing nothing (this is the "waste tube") and the second tube with 60 μl of ddH_2O in the cap. Make sure to load tubes without touching the inside of the tube or the cap to prevent contamination.
3. Check the collection tube containing the water for contamination at 5× magnification; contamination will be obvious as a fiber/hair or smaller particles floating on the water surface (*see* **Note 10**).
4. Locate your region of interest on the LCM slides at 5× magnification and switch to 10× magnification (plaques) or 20× magnification (NFTs) for LCM (Fig. 1).

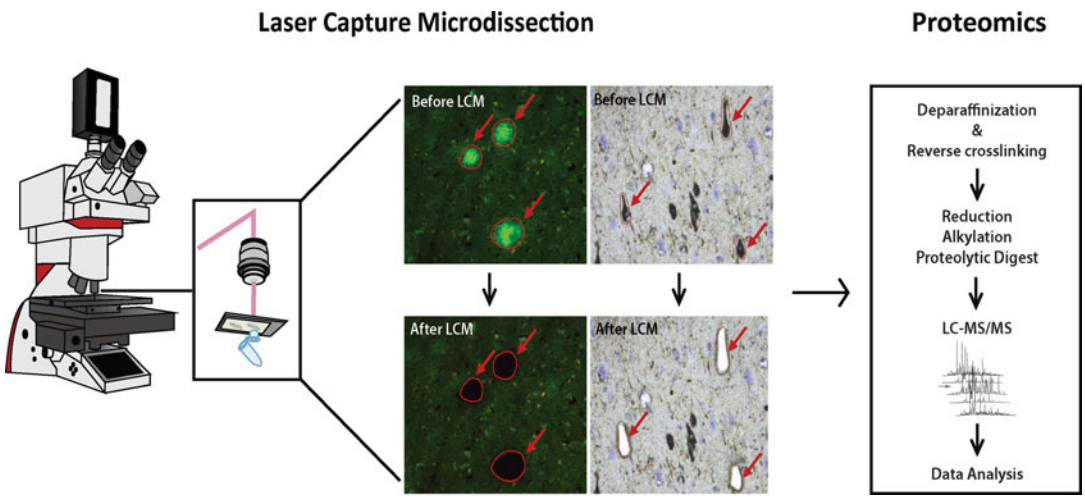

Fig. 1 Schematic of the method used to perform localized proteomics of plaques and neurofibrillary tangles. Aβ plaques and NFTs are microdissected using a LMD6500 microscope (Leica), which uses a precise laser to microdissect regions of interest. Microdissected plaques or NFTs fall into the collection cap by gravity. Representative images before and after LCM show the precision of LCM to isolate plaques (arrows; fluorescent green staining) and NFTs (arrows; brown staining). The plaques and NFTs proteomes are then identified and quantified using LC-MS

5. Position the waste collection tube under the slide and calibrate the laser using the "calibrate" function. It is important to calibrate the laser at the magnification that you will use to do LCM (in this case 10× or 20× magnification).
6. Optimize laser settings (*see* **Note 11**).
7. Position the second collection tube (contains water) under the slide, in preparation for LCM.
8. Outline and microdissect plaques or NFTs (*see* **Notes 12** and **13**).
9. Repeat **step 8** until at least 2 mm^2 of plaques and 1.5 mm^2 total area of NFTs have been microdissected (approximately 750 plaques and 4000 NFTs) (*see* **Notes 14** and **15**).
10. Remove collection tube carefully from the holder, making sure not to touch the rim or the inside of the tube in the process. Firmly close the cap of the collection tube.
11. Spin down the collection tube at 14,000 × *g* for 2 min to transfer the water and the microdissected plaques/NFTs to the base of the tube.
12. Store the sample at −80 °C until sample preparation for LC-MS.

3.5 Formic Acid Solubilization

1. Thaw the sample tubes at room temperature. Add 50 µl of 100 mM ammonium bicarbonate 20% acetonitrile solution to the cap of the tube, pipet up and down 2–3 times and centrifuge the sample to collect solution at the bottom of the tube. Repeat this step two more times. If one sample is dissected into multiple tubes, then combine the samples into one tube as described in **steps 2–4** (*see* **Note 16**).
2. Add 50 µl of 100 mM ammonium bicarbonate, 20% acetonitrile solution to the cap of each tube. Pipette up and down 2–3 times making sure the cap is thoroughly washed.
3. Close the cap and spin down the tubes to collect the solution containing LCM dissected cells at the bottom of the tube. Transfer the solution from the two tubes to one.
4. Add another 50 µl of 100 mM ammonium bicarbonate, 20% acetonitrile solution into the sample tubes 1b and 1c. Vortex the tubes and transfer the solution from tubes 1b and 1c to 1a.
5. Incubate the sample at 95 °C for 1 h, followed by incubation at 65 °C for 2 h in a preheated heat block to deparaffinize and reverse crosslink the proteins (*see* **Note 17**).
6. At the end of incubation cool the sample to room temperature. Spin the sample at 14,500 × *g* for 3 min to collect the condensed solution in the cap into the tube.
7. Put the sample in a SpeedVacc concentrator to dry.

8. Prepare a fresh solution of 70% Formic acid (LC-MS grade) by dissolving it into water (LC-MS grade) (*see* **Note 18**).
9. Add 90 µl of formic acid to the dried sample, vortex well and incubate overnight at room temperature.
10. At the end of incubation sonicate the samples for 5 min using a water bath sonicator with vortexing three times in between (*see* **Note 19**).
11. Completely dry the samples using a SpeedVac concentrator (*see* **Note 20**).

3.6 Reduction-Alkylation and Digestion

1. Resuspend the sample into 150 µl of 100 mM Ammonium bicarbonate solution, pH 8.0.
2. Prepare a fresh solution of 0.2 M DTT by dissolving 3 mg of DTT in 100 µl of ammonium bicarbonate. Add 2 µl of 0.2 M DTT solution to the sample and vortex gently (*see* **Note 21**).
3. Incubate the samples in a thermomixer at 57 °C for 1 h.
4. During the last 5 min of reduction, prepare a fresh solution of 0.5 M iodoacetamide by dissolving 9 mg of iodoacetamide in 100 µl of 100 mM ammonium bicarbonate and cover the tube with a foil to avoid light exposure (*see* **Note 22**).
5. Add 2 µl of 0.5 M iodoacetamide solution to the sample tube and vortex gently. Keep the samples at room temperature in the dark for 45 min.
6. Dilute the stock of sequencing grade-modified trypsin to a final concentration of 50 ng/µl using 100 mM ammonium bicarbonate. Add 4 µl of diluted trypsin to the sample. Pipette up and down to mix the enzyme into the solution and incubate the sample overnight at room temperature with gentle agitation.
7. Spin the sample at 14,500 × *g* for 1 min.
8. Quench the tryspin by adding trifluroacetic acid (TFA) at a 0.2% final concentration.
9. Add 15 µl of R2 POROS beads slurry to the sample. Incubate the sample at 4 °C for 2 h (*see* **Note 23**).
10. Place the C18 ZipTip onto an adaptor in a 1.5 mL Eppendorf tube.
11. Add 30 µl of LC-MS grade methanol and spin for 30 s using a bench top centrifuge at 1000 rpm, or until the solution passes through.
12. Repeat the **step 11** once using LC-MS grade acetonitrile and three times using 0.1% TFA.
13. Load 50 µl of the peptide sample to the C18 ZipTip and spin the sample at ~100–170 × *g*. Do not load more than 50 µl solution on a ZipTip at a time. G force should not be increased above 190 × *g*.

14. Repeat **step 13** until entire sample is loaded onto the C18 ZipTip.
15. Add 50 μl of 0.1% TFA to the empty sample tube. Vortex the sample to get residual POROS beads in the solution. Load the wash solution on the corresponding ZipTip and spin the sample for 30 s.
16. Repeat the **step 15** two more times.
17. Pipette 200 μl of 0.5% acetic acid solution into a fresh Eppendorf tube. To further thoroughly rinse the POROS beads in the ZipTip, aspirate 0.5% acetic acid through the ZipTip and pipette 8–10 times making sure the POROS beads move up and down (*see* **Note 24**).
18. Transfer the ZipTip on the top of a new 0.5 mL Eppendorf tube to elute the peptides. Make sure that the tube is labeled with a sample identifier.
19. Add 40 μl of 40% acetonitrile, 0.5% acetic acid solution to the ZipTip, and spin at 1000 rpm for 1 min to elute the peptides.
20. Add 40 μl of 80% acetonitrile, 0.5% acetic acid solution to the ZipTip and spin for 30 s at 1000 rpm to further elute the peptides.
21. Put the samples in a SpeedVac concentrator to remove organic solvent from the peptide mixture.
22. Reconstitute the dry samples in 15 μl of 0.5% acetic acid solution. Vortex the sample thoroughly.
23. Analyze 5 μl of the NFTs and 3 μl of AD plaque sample using LC-MS.

3.7 Mass Spectrometric Analysis

The samples were analyzed on LC-MS as described in [2] with some modifications described below.

1. One third of each sample was loaded onto the EASY spray 50 cm C18 analytical HPLC column with <2 μm bead size equilibrated in solvent A (2% acetonitrile, 0.5% acetic acid) using an EASY-nLC 1000 HPLC. The peptides were gradient eluted directly into the QExactive mass spectrometer (Thermo Scientific) using a 1 h gradient from 2% to 31% solvent B (90% acetonitrile, 0.5% acetic acid), followed by 10 min from 31% to 40% solvent B and 10 min from 40% to 100% solvent B (*see* **Note 25**).
2. The QExactive mass spectrometer was operated in data-dependent mode. The mass spectrometer acquired high-resolution full MS spectra with a resolution of 70,000, an AGC target of 1e6 with a maximum ion time of 120 ms, and scan range of 400–1500 m/z. Following each full MS, 20 data-dependent high-resolution HCD MS/MS spectra were

acquired using the following instrument parameters: AGC target of 5e4 with a maximum ion time of 120 ms, one microscan, 2 *m/z* isolation window, fixed first mass of 150 *m/z*, Normalized Collision Energy (NCE) of 27 and dynamic exclusion of 30 s (*see* **Note 26**).

3. The resulting MS/MS spectra were searched against the Uniprot Human reference proteome database using Sequest within Proteome Discoverer. Carbamidomethylation of cysteine was set as a fixed modification and oxidation of methionine as a variable modification. The mass tolerance was set to 10 ppm for both MS1 and MS/MS searches. FDR filtration was done at 1% FDR using a standard target-decoy database approach. Proteins identified with less than two unique peptides were excluded from analysis (*see* **Note 27**).

4 Notes

1. All the solutions should be prepared fresh and kept in clean, closed containers. Additionally, all surfaces, consumables, and containers should be clean and gloves should be worn throughout the process to prevent contamination from dust and other sources. This is because contaminating proteins (such as keratin found in dust) will interfere with and influence mass spectrometric analysis.
2. The archival time of FFPE blocks does not appear to significantly alter downstream LC-MS. To date, we have successfully performed LC-MS on tissue microdissected from FFPE blocks archived at room temperature from 1 to 15 years. Even though we have not yet examined tissue blocks that have been archived for longer than 15 years it is likely that such tissue is still amenable for the analysis given the lack of difference between blocks stored for 1 and 15 years.
3. The tissue has to be completely dry or it will detach from the slide during staining.
4. Xylene and ethanol solutions can be reused three times (30 slides total). Solutions should be stored in sealed containers when not in use to prevent contamination with dust.
5. Extra precaution is needed when staining on LCM slides as tissue sections do not adhere as strongly as they do to traditional glass slides. Therefore, tissue is more likely to detach during vigorous washes. In addition, use of detergents such as Tween-20 or Triton-X can result in tissue sections detaching from slides. Therefore, we recommend that these detergents should not be used. Also, all the reagents should be freshly made to prevent contamination.

6. All incubations and washes should be performed in an enclosed slide box to minimize photo-bleaching from this point onward.
7. Either fluorescent and peroxidase immunohistochemistry can be used to stain plaques and NFTs. Fluorescent immunohistochemistry has the benefit of a faster staining protocol that uses fewer reagents; however, all LCM then has to be done in the dark to prevent photo-bleaching. The benefit of peroxidase immunohistochemistry is that LCM does not have to be done in the dark (which is beneficial when microdissecting smaller regions such as neurons, as this is more time consuming). Importantly, both fluorescent and peroxidase immunohistochemistry are compatible with downstream LC-MS and we have not observed any proteomic differences when interchanging these techniques.
8. Using this protocol we incubate slides with DAB solution for 90 s. However, optimization studies should be performed for each antibody to determine the optimal incubation time that provides the greatest discrimination between staining and background.
9. The direction the tissue is facing is very important. LCM is still possible when the tissue is facing away from the collector; however, this appears to result greater static between the microdissected tissue and the underside of the slide, preventing some microdissected tissue from falling into the collector.
10. We use plastic wrap to cover the LCM microscope when in use. This prevents airflow between the slide and the collector, ensuring that microdissected plaques/NFTs fall into the collector. In addition it also prevents contamination from dust.
11. Ideally, you should manipulate the laser power, aperture, and speed settings so that the laser line is as thin as possible, while still maintaining a consistent cut that results in plaques/NFTs falling cleanly into the collector. Together, the power and the aperture determine the thickness of the laser line, but power should be carefully monitored because too much power results in tissue damage. A lower speed results in a cleaner laser cut; however, the speed should be optimized to be as fast as possible to minimize LCM time. Specimen balance helps to ensure that plaques/NFTs fall cleanly into the collection tube; this function provides an extra boost of power at the end of the cut, which helps plaques/NFTs to detach cleanly from the tissue section. We have noticed that some tissue is more friable than others. The exact reason for this is unknown, but it could result from different formalin fixation times, length of time between staining and LCM or tissue archival time. Therefore, it is best to check that the laser settings are optimal for each tissue section.

12. Microdissection of plaques identified using fluorescent immunohistochemistry must be performed in the dark to prevent photo-bleaching.
13. In our experience, it is best to limit the combined area for one continuous cut to ~100,000 μm^2 for plaques and ~50,000 μm^2 for NFTs. This is because the LCM stage position memory gets progressively more altered as more fields of view are included, which could result in non-plaque or non-NFT tissue areas being collected.
14. We performed optimization experiments to show that microdissection of at least 1.5 mm^2 total area from 8 μm thick sections is required for consistent downstream LC-MS [2]. A greater area results in marginally more comprehensive LC-MS results; therefore, 2 mm^2 area was microdissected for plaques. However, the additional time required to microdissect more NFTs was not justified by slightly better proteomic results; therefore, 1.5 mm^2 area was collected for these samples to minimize LCM time.
15. Dissection of 2 mm^2 of plaques takes approximately 2 h of continuous LCM time, which can be done in one session. However, LCM of 1.5 mm^2 NFTs takes approximately 8 h. This length of time is problematic because the water in the collection cap evaporates after approximately 6 h. We have found that using dry collection tubes results in significantly reduced protein identification using LC-MS, possibly due to increased adhesion between the microdissected tissue and the collection cap. Therefore, we recommend that NFT samples are collected into three separate tubes, each containing ~500,000 μm^2 total area, which are then combined prior to peptide extraction. The number of tubes that will be combined prior to peptide extraction should be consistent across all samples used in an experiment.
16. As discussed in Drummond et al. (2017) [13], the type of tubes used for LCM collection is critical as microdissected tissue can adhere to the collection cap, and the extent of this adhesion depends on the type of tube used. This is primarily a concern when analyzing microdissected single cells (e.g., NFTs) in comparison to larger tissue areas (e.g., amyloid plaques) as smaller microdissected areas are more likely to adhere to the collection cap. The LCM requires flat-cap 0.5 ml tubes, and only specific types of tubes physically fit into the LCM collector. While optimizing this method, we compared the effect of adhesion and consequent peptide identification using three different types of tubes (supplied by Biozym, Axygen, and Thermo Fisher). In our experience the tissue collected in Biozym and Axygen tubes yielded a larger number of

proteins identified in comparison to the Thermo Fisher tubes. Therefore, we recommend that investigators conduct preliminary experiments to evaluate the suitability of collection tubes used prior to large-scale experiments. Due to the limited availability of Biozym tubes, we recommend Axygen™ PCR tubes with 0.5 ml flat cap, which are a comparative alternative in our hands. We also recommend washing tubes prior to use with methanol followed by acetonitrile and three final washes with water (all LC-MS grade) to minimize keratin and polymer contamination.

17. Paraffin and intact crosslinks in the tissue will be detrimental for the downstream LC-MS analysis. The simple heating steps ensure that any residual paraffin is removed and it also reverses the formaldehyde crosslinks sufficiently to facilitate analysis by LC-MS.
18. Amyloid plaques and NFTs are primarily composed of aggregates of beta amyloid and tau respectively. The use of 70% formic acid is essential to solubilize aggregated proteins (particularly fibrillized Aβ and tau).
19. Only use water bath sonication. Use of probe type sonicators will result in protein loss, which cannot be afforded when using such a small amount of starting material.
20. The pH of the sample has to be 7.5 to allow successful reduction and alkylation by DTT and iodoacetamide, respectively, and digestion by trypsin. Always verify that the pH is 7.5 before proceeding with the alkylation and digestion step.
21. DTT denatures protein by reducing the disulfide bonds of proteins and thus facilitates complete digestion and improved protein coverage by LC-MS. The pH of the reaction should be 7.5–8.0. Alternative reducing agents, for example tris(2-carboxyethyl)phosphine (TCEP), can be used as well. This reagent works at lower pH, however, we found no difference when using TCEP versus DTT.
22. Alkylation of free cysteines prevents re-forming of cysteine disulfide bonds during the digestion steps. Disulfide bond formation would prevent detection of these peptides in the sample using standard search algorithms. Iodoacetamide is light sensitive. We recommend that iodoacetamide is prepared directly prior to use and that the reaction is performed in the dark. The pH of the alkylation reaction should be between 7.5 and 8.0 to ensure complete derivatization of cysteines while minimizing alkylation of other side chains. The concentration of iodoacetamide should be about 2.5 times the amount of DTT as each DTT molecule has two reaction sites for iodoacetamide. An excess of iodoacetamide or higher pH can cause alkylating lysine side chains, N-termini, methionine, histidine,

aspartate, and glutamate. This can reduce the sensitivity in the LC-MS analysis as the individual peptide signal is split over the different alkylated forms.

23. This step is performed to desalt the samples. Salts in the sample interfere with the ionization process and mass spectrometric detection. This leads to reduced sensitivity, which results in compromised peptide detection. C18 reverse phase columns are most commonly used for peptide desalting. Instead of POROS beads any C18 reverse material (i.e., stage tips or spin columns) can be used as well.
24. If you are using alternative C18 clean-up methods such as stage tips or spin columns, ensure efficient desalting by adding extra wash steps with 0.5% acetic acid.
25. Using a Q Exactive or Lumos mass spectrometer with 50 cm Easy spray columns and a 1 h gradient is sufficient for comprehensive detection of plaque and NFT proteins. Longer acquisition time did not significantly affect the number of proteins identified. However, the acquisition time and HPLC gradient should be optimized for each sample type.
26. The mass spectrometer acquisition parameters should be optimized based on the MS system being used. Generally, these samples are much less complex and contain less total protein than the typical proteomics sample. While for a typical proteomics experiment everything is optimized with speed in mind, care should be taken here to ensure sufficient ion signal for the MS/MS spectrum.
27. Even though the formaldehyde crosslinks are reversible, the workflow should be monitored for efficient reversal by checking for formylation on amino acid side chains, missed cleavages due to formylation on lysine residues that prevent cleavage by trypsin, as well as oxidation of methionine. We found the level of formylation in all tissues that we examined to be negligible, but we always include oxidized methionine as a reversible modification during data searching.

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Chapter 19

Cell-Specific RNA Quantification in Human SN DA Neurons from Heterogeneous *Post-mortem* Midbrain Samples by UV-Laser Microdissection and RT-qPCR

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Abstract

Cell specificity of gene expression analysis is from particular relevance when the abundance of target cells is not homogeneous in the compared tissue samples, like it is the case, e.g., when comparing brain tissues from controls and in neurodegenerative disease states. While single-cell gene expression profiling is already a methodological challenge per se, it becomes even more prone to artifacts when analyzing individual cells from human *post-mortem* samples. Not only because human samples can never be matched as precisely as those from animal models, but also, because the RNA-quality that can be obtained from human samples usually displays a high range of variability. Here, we detail our most actual method for combining contact-free UV-laser microdissection (UV-LMD) with reverse transcription and quantitative PCR (RT-qPCR) that addresses all these issues. We specifically optimized our protocols to quantify and compare mRNA as well as miRNA levels in human neurons from *post-mortem* brain tissue. As human *post-mortem* tissue samples are never perfectly matched (e.g., in respect to distinct donor ages and RNA integrity numbers RIN), we refined data analysis by applying a linear mixed effects model to RT-qPCR data, which allows dissecting and subtracting linear contributions of distinct confounders on detected gene expression levels (i.e., RIN, age). All these issues were considered for comparative gene expression analysis in dopamine (DA) midbrain neurons of the *Substantia nigra* (SN) from controls and Parkinson's disease (PD) specimens, as the preferential degeneration of SN DA neurons in the pathological hallmark of PD. By utilizing the here-described protocol we identified that a variety of genes—encoding for ion channels, dopamine metabolism proteins, and PARK gene products—display a transcriptional dysregulation in remaining human SN DA neurons from PD brains compared to those of controls. We show that the linear mixed effects model allows further stratification of RT-qPCR data, as it indicated that differential gene expression of some genes was rather correlated with different ages of the analyzed human brain samples than with the disease state.

Key words UV-laser microdissection, Single cells, Real-time quantitative PCR, Reverse transcription, *Post-mortem* human tissue, Non-optimally matched human samples, RNA integrity number RIN, Random primer, Oligo-dT primer, miScript, microRNA, Linear mixed effects model, Parkinson's disease, Dopamine

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https://doi.org/10.1007/978-1-4939-7558-7_19,

1 Introduction

Quantitative real-time PCR of reverse transcribed RNA (RT-qPCR) is still a gold standard for gene expression analysis of desired target genes [1, 2]. Also for global comparison of gene expression, e.g., via RNA-Seq and related techniques, single-cell resolution is a desired goal [3–7]. Transcriptional cell-to-cell variations—depending on cell cycle, epigenetics or unknown, potentially stochastic events—as well as disease-related variations might be masked if probes are not sampled on the level of identified individual cells [8, 9]. Furthermore, not only the differential expression of target genes might vary between control and disease samples, but also the composition of the tissue itself. In neurodegenerative diseases for instance, high levels of cellular heterogeneity, selective neuron loss, and disease-related changes in non-neuronal cells will contribute to an altered composition of the diseased brain tissue compared with that in control brains [10–12]. This will confound conclusions about specific gene expression changes in the cell type of interest.

The second most common neurodegenerative disease, Parkinson's disease (PD), provides a concrete example: one of the key pathological hallmarks of PD brains and its animal models is the substantial loss of dopamine containing (DA) midbrain neurons within the *Substantia nigra pars compacta* (SN) [13–16]. In PD, clinical motor symptoms manifest only when already about 75% of these DA midbrain neurons—the most prominent cell type within the SN—are lost [17, 18]. This massive loss of SN DA neurons will confound mRNA expression analysis of PD midbrain tissue when compared to tissue samples from control non-PD brains, simply because the number of SN DA neurons in PD and control midbrain tissue samples varies immensely. Furthermore, gene expression analysis at the level of PD midbrain tissue will be distorted by the altered numbers and functional states of non-neuronal cells like microglia, astrocytes, and local T-cells, known to change in PD [19]. And finally the midbrain contains different types of DA neurons that are affected differentially by degeneration in PD: while SN DA neurons are highly vulnerable to PD-stressors, neighboring DA midbrain neurons in the ventral tegmental arear remain largely resistant [20]. All these factors could explain the large number of different and even contrary findings of tissue-based gene expression studies in PD brains; e.g., for α-synuclein [reviewed in [21]]—a gene that can cause familial forms of PD when mutated (PARK1) or duplicated/triplicated (PARK4) [22–25]. Cell-specific quantification of gene expression with single-cell resolution overcomes these tissue-related limitations of gene expression data from pathological tissues and controls, since it enables the unbiased detection of cell-specific transcriptional dysregulation [26, 27].

Single-cell next-generation sequencing is a powerful tool to compare large numbers of single cells and to identify candidate genes, which show a dysregulated expression profile in disease conditions [28, 29]. However the large numbers of sample cells that are necessary for robust biologically meaningful results might not always be attainable. Likewise, isolated vital neurons might not be available, especially when only *post-mortem* human tissue is accessible. Therefore, the controlled isolation of desired cells with a subsequent specific analysis of identified target genes provides an alternative robust and specific experimental approach. Contact-free UV-laser microdissection (UV-LMD) is an ideal dissection method to isolate rare cell types from fixed *post-mortem* tissues (Fig. 1). In combination with reverse transcription and subsequent quantitative PCR (RT-qPCR)-based mRNA analysis of homogeneous cell pools and individual cells, provides such a candidate gene-based approach [30–37]. Here, we describe our most actual and detailed protocols for combining UV-LMD with RT-qPCR. We have specifically tailored and optimized these protocols to quantify and compare mRNA as well as miRNA levels in human SN DA neurons from *post-mortem* midbrain tissue samples of PD patients and unaffected controls, utilizing either a random primer-based reverse transcription strategy, or an oligo-dT primer-based approach, followed by qPCR (Fig. 2). Non-optimally matched samples (e.g., distinct

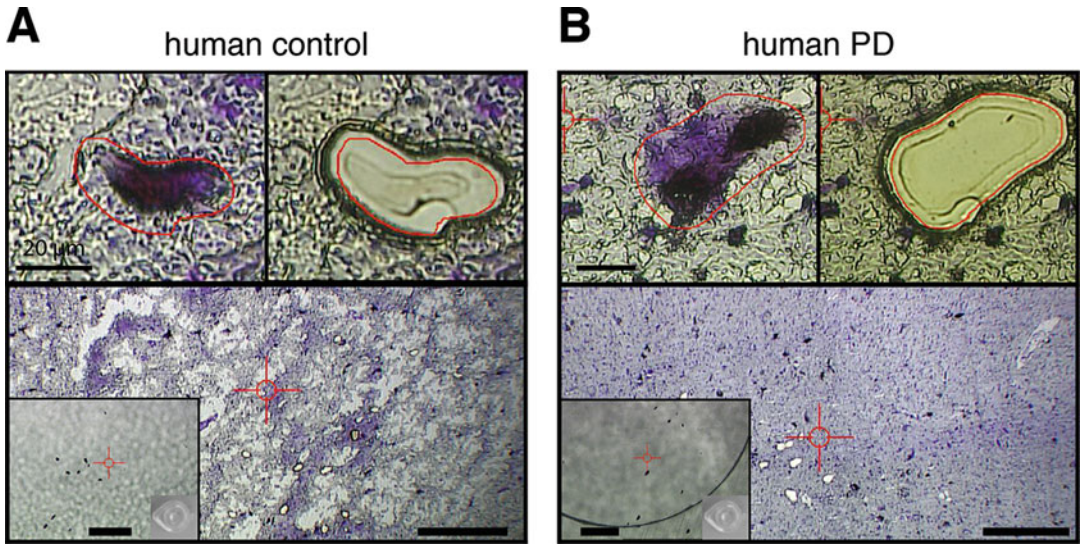

Fig. 1 UV-laser microdissection (UV-LMD) of individual neuromelanin-positive SN DA neurons of human control (**a**) and Parkinson's disease, PD (**b**) cresylviolet-stained tissue sections. *Upper row*: individual neurons before (left) and after UV-LMD (right). Scale bars: 20 µm. *Lower row*: overview of the horizontal midbrain section containing the *Substantia nigra* after UV-LMD of 15 individual SN DA neurons. Please note the higher integrity of the PD tissue (presumably due to well-described reactive gliosis). Scale bars: 500 µm. *Inserts*: inspection of the reaction tube cap for validation of successful collection of all laser microdissected SN DA neurons. Scale bars: 400 µm

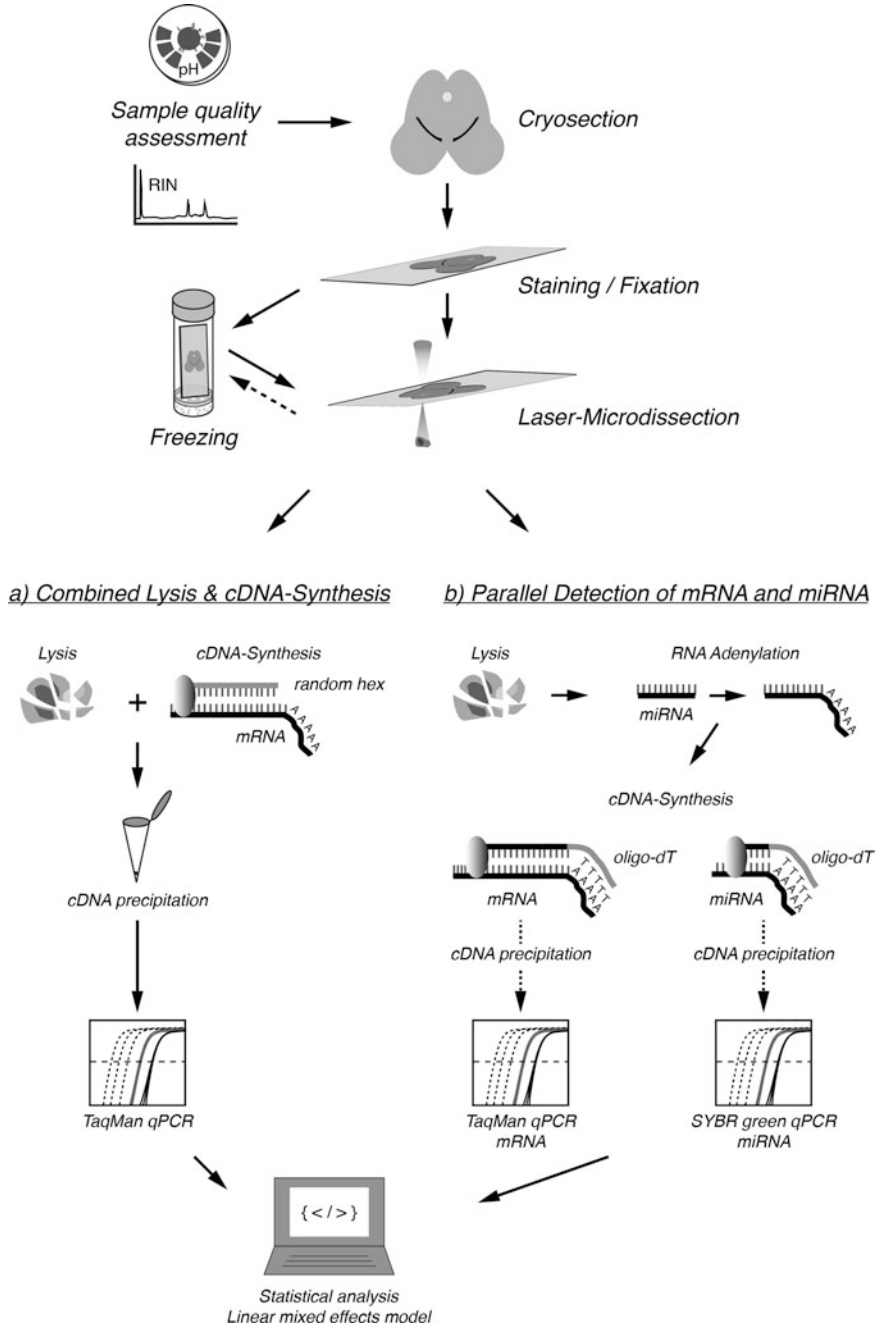

Fig. 2 Flowchart illustrating the general experimental procedure for UV-laser microdissection (UV-LMD) and RT-qPCR-based mRNA and miRNA gene expression analysis of individual human SN DA neurons from *post-mortem* midbrains of PD patients and controls. For details, please see the text. RIN = RNA integrity number

donor ages) and different, non-optimal RNA integrities (quantified via the RNA integrity number, RIN [38]) of individual human samples are a common problem for gene expression studies of human tissue samples [39–45]. Consequently, we have not only

refined our RT-qPCR protocols, but also our data analysis, by applying an optimized linear mixed effects model to our human SN DA neuron derived UV-LMD RT-qPCR data (Fig. 2). This model allows dissecting linear contributions of distinct confounders on detected gene expression levels (i.e., distinct RINs of tissue samples, distinct ages of donors, distinct post-mortem intervals, PMIs). By comparing SN tissue-based and SN DA neuron-specific RT-qPCR results from human PD and control brains, we demonstrate that detected tissue-based gene expression differences most likely will not reflect the differential gene expression of SN DA neurons from PD and control brains [46].

With the here-detailed protocols, we identified that a variety of specific genes—coding, e.g., for ion channels, for dopamine synthesis, reuptake and packaging proteins, and for PARK gene products—display a transcriptional dysregulation in remaining human SN DA neurons from PD brains compared with those of controls [34, 37, 46–51]. These findings contribute to a better understanding of the SN DA neuron-specific pathophysiological process in PD. The identified cell-specific transcriptional dysregulations in human SN DA neurons in PD were not correlated with a respective downregulation of the miRNA miR-133b [46], as suggested by human SN tissue-based approaches [52]. We further illustrate that the application of our linear mixed effects algorithm allows further stratification of human SN DA neuron-derived RT-qPCR data, as it strongly suggests that differential gene expression of some genes (e.g., the vesicular monoamine transporter) VMAT2 is likely rather correlated with different ages of the individual analyzed human brains than with their disease state, while significantly lower mRNA levels of the transcription factor NURR1 in SN DA neurons from PD brains became evident after data stratification for distinct donor-ages and RIN of brain-samples (Fig. 3). In summary, we provide here our most actual step-by-step protocols for comparative single-cell gene expression profiling of human *post-mortem* PD and control brains, by combining UV-laser microdissection and RT-qPCR techniques that are specifically tailored for quantification of mRNA and miRNA levels in SN DA neurons from *post-mortem* human midbrains. However, most considerations and approaches are also applicable for non-candidate-gene-driven expression profiling approaches, like single-cell RNA-Seq after global RNA/cDNA amplification.

Analyzing cell-specific mRNA/miRNA levels in human *post-mortem* PD and control brains provides a particular challenge (in contrast, e.g., to analyzing perfectly matched mouse brain cohorts), due to two inevitable reasons:

1. Inevitably, the human brain samples will not be perfectly matched. Besides age, gender, and disease state, each human individual has its own genetic background and its own specific

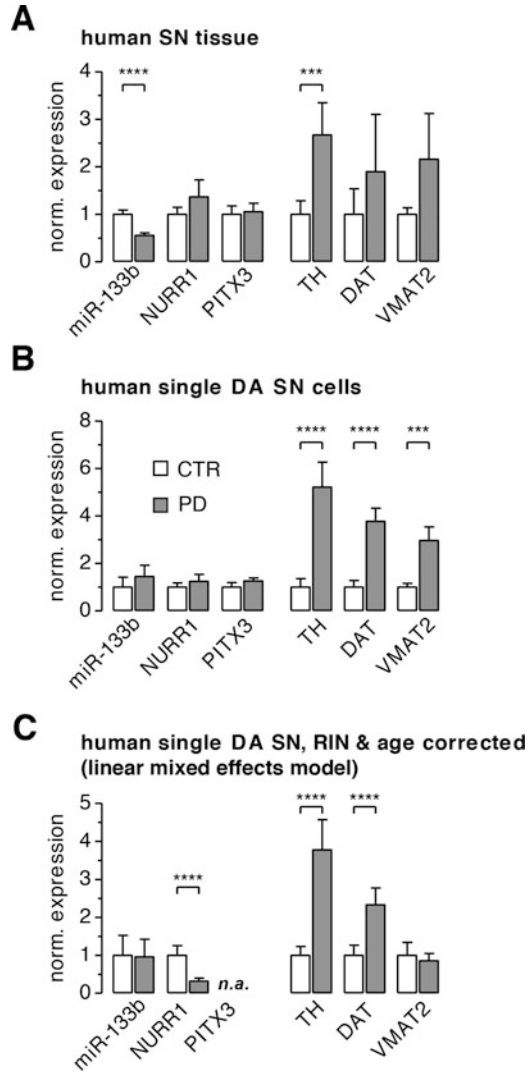

Fig. 3 Elevated mRNA levels of dopamine release genes but not of miR-133b in SNCA-overexpressing SN DA neurons in sporadic PD. (**a**) Levels of miR-133b and mRNAs for genes involved in DA neuron development/maintenance (NURR1, PITX3) and dopamine synthesis, reuptake and vesicular packaging (TH, DAT, VMAT2), determined via RT-qPCR at the level of midbrain tissue from PD brains and controls. Tissue RT-qPCR data are normalized to a geometrical mean of β-actin, ENO2 (neuron-specific enolase) and the transcription initiation factor TIF-1A, and are given normalized to control brain levels. Note significantly lower expression of miR-133b and significantly higher levels of only TH in PD SN tissue compared to controls. (**b**/**c**) RT-qPCR analysis of genes as in (**a**), but at the cell-specific level of individual SN DA neurons, without (**b**) and after (**c**) linear mixed effects model data analysis and adjustment of data for RIN and age effects (data are given normalized to controls). (**b**) Note that miR-133b levels are not altered in SN DA neurons from PD compared to controls. These results identify the miR-133b downregulation in (**a**) and as described in [52] as a tissue-artifact, caused rather by the loss of SN DA neurons in SN-tissue in PD. These data emphasize the importance of cell-specificity when comparing gene expression in *Substantia nigra* from PD and control brains, with variations in the number of SN DA target cells due to disease state. (**c**) Note that mathematical adjustment of cell-specific data for age and RIN effects of NURR1 expression (linear mixed effects model) suggests a

life-long environmental and medical history [53–55]. Even if the provided human samples might appear well matched, they will always have a higher intrinsic heterogeneity than, e.g., respective mouse cohorts.

2. Most likely, the human brain samples will display differential degrees of RNA-degradation, depending on the circumstances of death and *post-mortem* brain removal [39–41]. In most cases, the experimenter cannot control this. It is nevertheless particularly important to avoid further degradation of RNA due to sample handling and experimental procedures.

We evolved two strategies to address these issues. First, given that our human brain samples displayed variable RIN values [56], we empirically tested that both our utilized RT-qPCR protocols were not affected by different RNA qualities, by using differently degraded RNA as RT-qPCR templates (Fig. 4). We utilized the same amounts of the same RNA as RT-qPCR templates, however with different RNA-integrities (RIN between 5.9 and 9.9, covering the range of RIN values of the human brain samples that were available to us). RT-qPCR analysis showed similar results for all the samples, unaffected by different RIN values for both RT-qPCR protocols (mouse LDH-2 assay). More importantly, both RT-qPCR protocols resulted in similar differential expression values for the PARK1/4-gene product α-synuclein for individual human SN DA neurons between PD and control brains, either if reverse transcribed with the random hexamer protocol or with the miScript oligo-dT primer-based protocol. Note that the first set of experiments and the respective third set were carried out 3 years apart (storage and re-use of human midbrain sections at −80 °C within that time). For further details *see* [46].

Second, to minimize the bias resulting from non-optimal matched tissue cohorts, we developed a model-based mathematical strategy for data analysis and stratification. After implementing a linear mixed effects model [57, 58], we dissect and subtract

Fig. 3 (continued) downregulation of NURR1 mRNA in PD (masked by a model-suggested linear mRNA upregulation with age). Furthermore, the cell-specific elevated mRNA levels of VMAT2 in SN DA neurons in PD are not preserved after model adjustment for RIN and age effects, as VMAT2 mRNA levels of different brains are well represented by a linear age dependence, rather than by disease state. Note that model results were not reliable for PITX3 data, as the algorithm did not converge well, likely due to low numbers of samples with positive RT-qPCR results. These data highlight the power (and limitations) of the linear mixed effects model analysis of the RT-qPCR data, as the analyzed human PD and control samples were not perfectly age matched (compare Fig. 3a). Bar graphs represent normalized expression as mean ± S.E.M., asterisks indicate significant differences (***t-test, p-value $\leq$0.0001; **** p-value $\leq$0.00001); all data adapted from [46], for details see the text and there. *CTR* control (white bars), *PD* Parkinson's disease (grey bars), *TH* tyrosine hydroxylase, *DAT* dopamine transporter, *VMAT2* vesicular monoamine transporter 2, *ENO2* neuron specific enolase 2, *NURR1* nuclear receptor related 1 protein, *Pitx3* pituitary homeobox 3

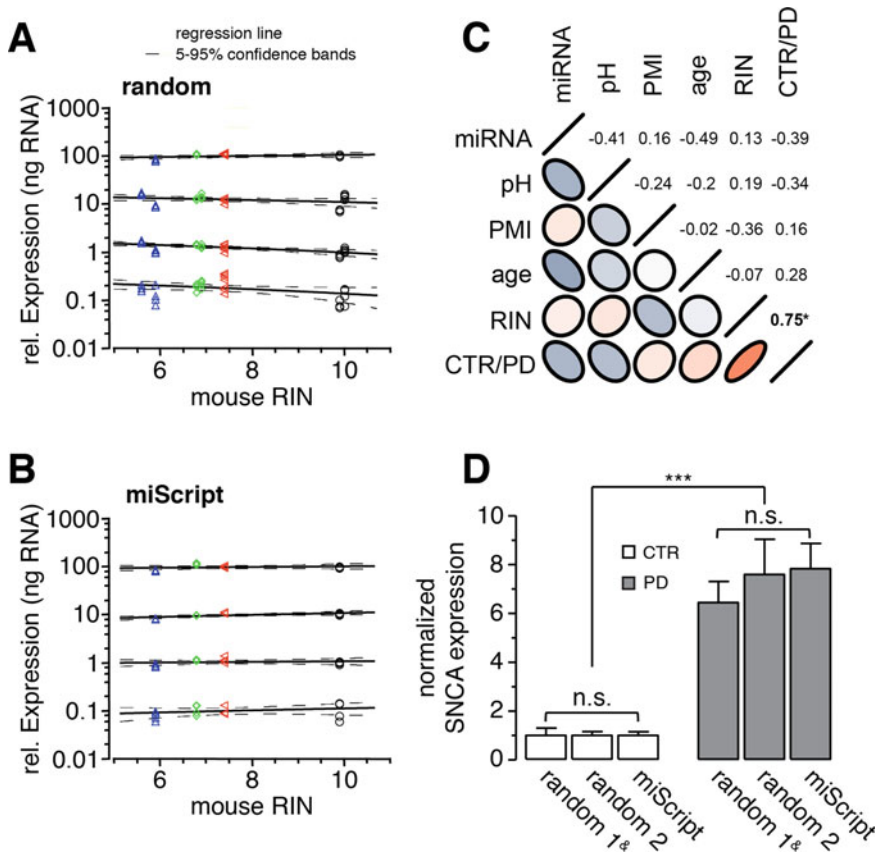

Fig. 4 Experimental evaluation of robustness and reproducibility of two different RT-qPCR protocols for mRNA/miRNA quantification in human SN DA neurons from *post-mortem* PD and control brains. (**a**, **b**) Sensitivity and reproducibility of two different, optimized RT-qPCR protocols (**a**: random hexamer primer-based protocol; **b**: oligo-dT primer-based miScript protocol) is independent of integrity levels of RNA used as templates for cDNA synthesis (RIN range: 9.9–5.7 of mouse cDNA, covering the RIN-spectrum of the respective human samples of this study, **compare c**; qPCR assay: mLdh-2). cDNA samples with different RINs were generated by thermal degradation of the same mouse midbrain tissue derived RNA for 0–72 min at 70 °C. Results of RIN 9.9 standard curves were used for the calculation of relative expression levels of mLdh-2 at different RIN values (range: 9.9–5.9). Regression lines with confidence bands show no significant dependence of gene expression levels from RIN values at all dilutions. (**c**) Characterization and comparison of the distinct human *post-mortem* brain samples, analyzed in this study. Partial correlations between two given parameters were controlled by the other four parameters in each calculation. Shape of ellipses and color define strength of correlation (red: positive correlation, right leaning ellipse; blue: negative correlation, left leaning ellipse). Asterisk indicates significance; $p = 0.75$. Note the particularly strong and significant correlation between RNA quality (given as RNA integrity number RIN) and disease state (control CTR vs. Parkinson's disease, PD), due to significant difference of RIN values between control brains (CTR, $n = 8$) and PD brains ($n = 5$). *RIN* RNA integrity number, *PMI post-mortem* interval, *miRNA* microRNA. (**d**) Using the oligo-dT primer-based miScript RT-qPCR protocol, similar degrees of significantly elevated alpha-synuclein (SNCA) mRNA levels were detected in remaining human SN DA neurons from PD brains compared to those of controls, as with the random hexamer based RT-qPCR protocol. Random 1 data adapted from [48], random 2 as well as miScript data adapted from [46], for details see the text and there. Bar graphs (mean ± S.E.M.) show normalized SNCA expression levels of three individual human SN DA sample sets from control brains (random 1, miScript 5: $n = 5$ brains; random 2: $n = 8$ brains) and PD brains ($n = 5$). Asterisks (***) indicate significant difference (*t*-test, *p*-value ≤ 0.0001)

possible linear influences of distinct confounders on RT-qPCR data—in our case distinct age—and RIN-values of individual brains, but the model is also applicable, e.g., to distinct *post-mortem* intervals (PMI) or tissue pH-values [46, 59].

2 Materials

2.1 Handling, Fixation, and Staining of Human Brain Tissue

1. To prevent RNase contamination, human brain tissue specimens are stored in heat-sterilized tinfoil and RNase-ExitusPlus (AppliChem) treated parafilm-sealed boxes at −80 °C.
2. Molecular biology grade, certified RNase-free H_2O and ethanol are used. Primer/reagent solutions as well as mastermixes are prepared with RNase-free H_2O supplied by 5 PRIME.
3. Microtome blades (Feather, Type R35) are rinsed two times for 30 s in 75% ethanol and whipped with RNase-ExitusPlus and RNAse-free isopropanol (Sigma) (*see* **Note 1**).
4. Ethanol dilution series (2× 75%, 95%, 100% absolute (Sigma) and one tube Ethanol anhydrous (Alfa Aesar, see below)) are freshly prepared with RNAse-free H_2O (Qiagen) on each experimental day and stored in 50 ml Falcon tubes at room temperature. One tube of 75% ethanol is kept at −20 °C. Ethanol anhydrous stock (90% ethanol, 5% methanol, 5% isopropanol, AlfaAesar) is stored with molecular sieve (Merck, pore size: 0.3 nm, 25 g/l) to avoid rehydration.
5. 1% Cresyl Violet (CV) acetate staining dye (Sigma) is diluted in 100% ethanol (Sigma), stored in a tinfoil covered and parafilm sealed Falcon tube, and incubated for at least 1 week before use, as CV dissolves non-optimal in ethanol.
6. A RNase-free drying box with Silica gel with moisture indicator (Merck) is used.
7. pH values of human *post-mortem* midbrain tissue samples are analyzed with a pH Optica micro system and a pH MicroTip Fiber Optic sensor, 140 μm OD (both WPI).
8. RNA integrity number (RIN) analysis, a measure for RNA degradation [56], is performed with the Agilent 2100 Bioanalyzer system. For mRNA integrity analysis the Agilent RNA 6000 Nano Chip Kit, and for analysis of small RNA amounts and % miRNAs the Agilent Small RNA Chip-Kit are used.

2.2 UV-Laser Microdissection

1. A contact-free UV-laser microdissection (UV-LMD) microscope is needed (this protocol was successfully tested with both, the Zeiss PALM UV-LMD setup and the Leica UV-LMD6000 and UV-LMD7000 setups; currently we only use the UV-LMD7000). Heat-sterilization (180 °C, 2 h) of all UV-LMD microscope parts that are in contact with the tissue

slides (i.e., slide holder) or reaction tubes (i.e., cap/tube holder, forceps) prevents RNase contamination (*see* **Note 1**).

2. PEN-membrane slides (MicroDissect, 2.0 μm) for mounting of tissue sections and laser microdissection are treated with UV-C light for 20 min.
3. RNase-free thin-walled 0.5 ml PCR reaction tubes with flat cap (Axygen PCR thin-wall, clear, 0.5 ml) are UV-C treated with an open cap for 45 min, before using them for cell collection, combined cell lysis and cDNA synthesis, and cDNA precipitation.

2.3 Cell-Lysis and Reverse Transcription

2.3.1 Preparation of Cap-Mix for Combined Cell Lysis and cDNA Synthesis

1. Cell lysis and cDNA synthesis are performed in the same buffer (*Cap-Mix*) containing 0.5% NP-40 (Roche, light sensitive, stored as 10% stock in aliquots in the dark at +4 °C), 5 U SUPERase-In (Thermo Fisher Scientific, stored in aliquots at −20 °C), 0.5 mM dNTPs (GE Healthcare, stored as 20 mM stock at −20 °C), 5 μM random hexamer primers (Roche, stored as 1 mM stock aliquots at −20 °C), 500 ng poly-inosine (Sigma, stored as 1 μg/μl stock at −20 °C), 2 mM Tris-HCl pH 7.4 (Sigma, 100 mM stock stored at −20 °C), 10 mM DTT (Thermo Fisher Scientific, stored as 100 mM stock at −20 °C) in 1× first-strand buffer (Thermo Fisher Scientific, 5× stock: 250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCl_2$, pH 8.3, stored in aliquots at −20 °C) at a final volume of 4.7 μl per reaction.
2. *Cap-Mix* sufficient for the number of samples that are collected (plus positive and negative controls) is freshly prepared on each experimental day (*see* **Note 2**) and stored on ice in a light-protected, RNase-free 0.5 ml single sealed reaction tube (Eppendorf biopure, tube shaft and lid covered with tube labels). All the components are carefully added, mixed by finger flipping and quickly centrifuged. SUPERase-In is added directly from −20 °C to the reaction mix. Poly-inosine, NP-40, and SUPERase-In are viscous and special care has to be taken during pipetting to avoid air bubbles or volume errors. If bubbles have formed, the mix is centrifuged briefly until all bubbles disappear (*see* **Notes 3** and **4**).
3. 60 U (=0.3 μl) SuperScript II Reverse Transcriptase (Thermo Fisher Scientific, stored in aliquots at −20 °C) are added to each reaction after lysis (*see* Subheading 3.3). The enzyme aliquots are stored in a benchtop freezer (Techne) at −20 °C during the experiment to avoid "freeze-thaw-cycles."

2.3.2 Preparation of Lysis Mix and miScript Buffer Mix for Cell Lysis with Subsequent miScript Reverse Transcription

1. Cell lysis is performed in the *miRNA lysis mix* containing 0.5% NP-40 (Roche, light sensitive, stored as 10% stock in aliquots in the dark at +4 °C; final concentration after reverse transcription 0.25%), 10 U SUPERase-In (Thermo Fisher Scientific, stored in aliquots at −20 °C), 500 ng poly-inosine (Sigma, stored as 1 μg/μl stock at −20 °C) at a final volume of 5 μl per reaction.
2. miRNA lysis mix sufficient for the number of samples that are collected (plus positive and negative controls) is freshly prepared on each experimental day as described above.
3. Parallel reverse transcription of mRNA and miRNA is performed by adding 4.5 μl of the *miScript buffer mix* (2 μl 5× *miScript RT buffer*, miScript Reverse Transcription Kit, Qiagen, and 2.5 μl RNase-free H_2O (5 PRIME)) and by the addition of 0.5 μl *miScript RT mix* (containing polyadenylase and reverse transcriptase, miScript Reverse Transcription Kit, Qiagen) in a final volume of 10 μl per reaction.

2.4 cDNA Precipitation

Depending on the subsequent processing of the cDNA, a purification step via ethanol precipitation is recommended [60].

1. cDNA precipitation should be performed in a reaction tube, which is suited for longer high-speed centrifugation. As cDNA precipitation of UV-LMD samples should best be performed in the same tube after reverse transcription, employing a reaction tube that is tested and established for both, UV-LMD and cDNA precipitation is strongly recommended.
2. cDNA precipitation is performed with *precipitation-mix* containing 1 μg glycogen (stored as 1 μg/μl stock-solution at −20°C, Thermo Fisher Scientific), 350 ng PolydC (stored as 1 μg/μl stock-solution at −20°C, Midland) and 1/10 volume sodium acetate (NaAc, i.e., 1.2 μl of 3 M stock-solution, pH 5.5, stored at room temperature, Thermo Fisher Scientific) added to 4.8 μl H_2O (5 PRIME) in a total volume of 7.3 μl.
3. cDNA *precipitation mix*, sufficient for the number of samples, plus positive and negative controls is freshly prepared on each experimental day. All the components are carefully added, mixed by vortexing, and quickly centrifuged. As precipitation control, serial cDNA dilutions for generation of a qPCR standard curve are precipitated and analyzed in respect to the respective non-precipitated cDNA standard curve.
4. Certified RNase-free ethanol is used for cDNA precipitation.

2.5 Quantitative Real-Time PCR

1. cDNA for the generation of standard curves (serial dilutions over four magnitudes, e.g., 30–0.03 ng) to assess assay performance is needed, e.g., human tissue SN cDNA (1 μg/μl,

derived from Human Brain, Substantia Nigra Total RNA, Clontech/TaKaRa).

2. A GeneAmp 7900HT real-time qPCR system (Applied Biosystems) or comparable instrument and 96-well PCR plates (Thermo Fisher Scientific) with optical adhesive film covers (Thermo Fisher Scientific) are needed.

2.5.1 TaqMan PCR Reaction Contents

1. 2× QuantiTect Probe qPCR Master Mix (Qiagen).
2. 20× TaqMan PrimerProbe Assay (Life Technologies, for details *see* **Notes 5** and **10**).

2.5.2 SYBRgreen PCR Reaction Contents (for miRNA Amplification)

1. 2× QuantiTect SBYR Green PCR Master Mix (Qiagen).
2. 10× miScript Universal Primer (sequence: company properties, Qiagen).
3. 10× miScript Assay (in 1× Tris-EDTA, pH 8, Qiagen, *see* **Note 10**).

2.6 Linear Mixed Effects Model

1. R project software for statistical computing (R-Development-Core-Team, 2012), Version 2.15.1 or later.
2. R-package ASReml-R (VTN International Ltd., Hemel Hempstead, UK).

3 Methods

To guarantee successful UV-LMD and subsequent gene expression analysis of small cell pools and individual cells, it is essential to work in a strictly RNase-free regime. For details on RNase-free working conditions, *see* **Note 1**. The protocol described below was used to quantify mRNA/miRNA levels in neuromelanin-positive SN DA neurons from human *post-mortem* midbrain tissue blocks, provided by the German BrainBank. An overview of the experimental procedures is illustrated in Fig. 1.

3.1 Storage, Cryosectioning, and Staining of Human Brain Tissue

1. On the experimental day, human brain tissue is transferred (on dry ice) from −80 °C to the quick-freeze panel of a precooled cryostat (−35 °C) and glued with tissue freezing medium (Leica) on a specimen holder (*see* **Note 6**). After an equilibration period of 20 min at −35 °C, the cryostat is set to the cutting temperature (for our specimens −19 °C), and additionally equilibrated for 45 min, before 12 μm horizontal midbrain sections including the SN are cut. Chippings from the trimming procedure are collected for RNA quality and tissue pH analysis (*see* Fig. 1 and **Note 7**).
2. The brain sections are mounted on UV-C treated PEN-membrane slides and allowed to thaw briefly. Once thawed, the slide is transferred to the Falcon tube with 75% ethanol at −20 °C and fixed for 2 min.

3. The slide is removed with a sterile forceps, and 0.25 ml 1% cresyl violet staining solution is applied directly on the slide via a sterile filter syringe (0.1 μm; Whatman), incubated for 1 min, then dipped briefly in 75%, 95% and 100% ethanol absolute and finally incubated for 1 min in ethanol anhydrous.
4. The fixed and stained slides, each containing several brain sections, are stored in a drying chamber containing silica gel for at least 45 min before UV-LMD.
5. Alternatively, after drying, the slides are long-term stored at −80 °C in storage jars, containing silica gel (*see* Fig. 1 and **Note 8**).
6. For human brain pH value analysis, tissue chippings (*see* **Note 7**) are homogenized in 4 °C cold H_2O (10 μl per mg tissue) with a sterile 1 ml syringe and a 21-gauge needle. The MicroTip fiber optic pH sensor is calibrated at 4 °C. Measurements are performed on ice or room temperature.
7. RIN values as well as amounts of small RNAs and % of miRNA are determined from RNA, isolated from human brain tissue chippings (*see* Fig. 1 and **Note 7**) via the RNeasy MINI kit (Qiagen), eluted in an volume of 30 μl H_2O (5 PRIME). 1.5 μl of eluted RNA is mixed with 5 μl of fluorescence marker and run on an Agilent RNA 6000 Nano Chip or the Agilent Small RNA Chip, respectively.

3.2 UV-Laser Microdissection of Individual Neurons from Human Brain Samples

1. All workspaces are cleaned to ensure RNase-free working conditions (*see* **Note 1**).
2. Slides with tissue sections are placed on the sterile slide holder and transferred to the UV-LMD microscope. Tissue quality and staining are inspected under low and high magnifications, and only sections that allow clear identification of individual cells are further processed (*see* Fig. 2). For the identification of individual human SN DA neurons, their brown-black neuromelanin content is very helpful.
3. Laser-settings need to be optimized for each individual section.
4. After the brain region of interest is identified (in our case SN), an UV-C treated thin-walled PCR reaction tube is placed in the cap holder and transferred to the microscope. The reaction tube cap is inspected with the *cap-control* function to exclude rarely occurring contaminations with dust particles. Individual SN DA neurons are cut and harvested into the cap of the reaction tube. It is highly recommended to visually control that all laser microdissected neurons were successfully harvested (*cap-control* function, *see* Fig. 2).

3.3 Lysis and cDNA Synthesis of Individual Laser Microdissected Neurons from Human Brain Samples with Random Hexamer Primers

For each set of single-cell experiments, suitable positive controls (e.g., cDNA; derived from commercially obtained human SN tissue RNA, *see* Subheading 2.5.1) and negative controls (no UV-LMD harvested cells in cap) are processed in parallel.

1. If the *cap-control* is positive, the cap holder is removed and 4.7 μl *Cap-Mix* are added to the cap immediately. Any direct contact between the cap and the pipette tip must be avoided.
2. The reaction tube is carefully removed from the cap holder and the tube is closed upside down to ensure that the *Cap-Mix* remains in the cap.
3. The reaction tube is placed upside down on the cap in a preheated (72 °C) thermoblock (ThermoStat, Eppendorf) and incubated for 2 min for cell lysis (*see* **Note 9**).
4. Afterwards the tube is transferred onto an ice-cold metal block, upside-down, and allowed to cool for 5 s.
5. The *Cap-Mix* is then spun down at 11,200 × *g* with a benchtop centrifuge (MiniSpin plus, Eppendorf) for 1 min at room temperature and transferred onto an ice-cold metal block to cool down for 1 min.
6. 0.3 μl SuperScript II are added directly to the *Cap-Mix* in the bottom of the tube.
7. The tube is transferred to a preheated (38 °C) thermomixer (Eppendorf, 350 rpm for 10 s every 10 min) and random hexamer-based cDNA synthesis is carried out overnight. For overnight incubation, after 2 h at 38 °C, all the samples are spun down briefly, and are transferred to a preheated thermobox (ThermoStat, Eppendorf) for final overnight cDNA synthesis (39 °C). After cDNA synthesis, the samples are stored at −20 °C until further processing.

3.4 Combined Lysis and cDNA Synthesis with Oligo-dT-Primers (miScript)

1. If the *cap-control* is positive, the cap holder is removed and 5 μl *miRNA lysis Mix* is added to the cap immediately. Any direct contact between the cap and the pipette tip must be avoided.
2. The reaction tube is carefully removed from the cap holder and the tube is closed upside down to ensure that the *miRNA lysis Mix* remains in the cap.
3. The reaction tube is placed upside down on the cap in a preheated (72 °C) thermobox (ThermoStat, Eppendorf), and incubated for 2 min for cell lysis (*see* **Note 9**).
4. Afterwards the tube is transferred onto an ice-cold metal block, upside-down, and allowed to cool for 1 min.
5. The *miRNA lysis Mix* is then spun down at 11,200 × *g* with a benchtop centrifuge (MiniSpin plus, Eppendorf) for 1 min at room temperature.

6. 4.5 µl *miScript buffer mix* and 0.5 µl *miScript RT mix* are added directly to the *miRNA lysis Mix* in the bottom of the tube after spinning.
7. The tube is transferred to a preheated (38 °C) thermomixer (Eppendorf, 350 rpm for 10 s every 10 min) and polyadenylation and cDNA synthesis are carried out for at least 2 h or overnight. After cDNA synthesis, the samples are stored at −20 °C until further processing.

3.5 cDNA Precipitation

1. 7.3 µl of the *precipitation-mix* are added directly to each 5 µl cDNA reaction into the cDNA reaction tube. The samples are vortexed thoroughly and briefly centrifuged.
2. About 3 volumes of 100% ethanol (40 µl, Applichem) are added. The samples are vortexed thoroughly and briefly centrifuged.
3. The samples are precipitated overnight at −20 °C.
4. Reaction tubes are centrifuged at 0 °C at 16,100 rcf for 2 h.
5. The supernatant of each sample is discarded via pipetting. Physical contact with the cDNA pellet is carefully avoided.
6. 100 µl of 80% ethanol (Applichem) are added (pellet should not be resuspended or vortexed), the samples are centrifuged for 15 min at 0 °C and the supernatant is again carefully discarded via pipetting.
7. cDNA pellets are dried in opened reaction tubes at 45 °C for 10 min in a ThermoMixer (Eppendorf).
8. The desired volume of H_2O (5 PRIME) is added. Contact of the cDNA pellet with the pipet tip is avoided. Tubes are closed, shortly centrifuged and incubated for 2 h at 45 °C in a ThermoMixer (Eppendorf) with interval shaking (550 rpm for 10 s every 10 min) to dissolve cDNA pellets. Samples are spun down at least each 40 min (three times).

3.6 Quantitative Real-Time PCR of UV-LMD cDNA Samples for mRNA and miRNA Quantification

1. The following procedures are carried out in a UV-C treated sterile workbench.
2. Best, precipitated cDNA is used (*see* Subheading 3.4). If cDNA is NOT precipitated, it has to be diluted at least tenfold to avoid inhibitory effects of the RT-reaction on the qPCR [60]. In this case, each single-cell cDNA sample is diluted by adding 50 µl (random hexamer samples) or 45 µl (miScript samples) molecular biology grade H_2O (5 PRIME).
3. Tubes are stored in ice-cold metal blocks.
4. A serial dilution of a cDNA standard (see above Subheading 2.5.1) is run in parallel with each experiment (*see* Subheading 2.5), as a PCR positive control, and for standard curve generation to assess assay performance and to calculate the cDNA amount of the UV-LMD samples in respect to the standard curve.

3.7 TaqMan PCR (for mRNA qPCR Amplification)

1. A mastermix for each individual gene of interest for all the samples for quantitative real-time PCR in 20 µl reactions is prepared by mixing 10 µl 2× QuantiTect Probe qPCR Master Mix, 1 µl 20× primer/probe mix (for gene of interest, for details **Notes 5** and **10**) and 4 µl H_2O (depending on the cDNA volume used) for each UV-LMD sample (volumes are multiplied by the number of samples + controls + 1).
2. 15 µl of respective mastermix is added to the bottom of a MicroAmp 96-well reaction plate. 5 µl of cDNA (adjust volumes accordingly if cDNA in more or less volume is used) is added to the mastermix and the plate is sealed with an *optical adhesive cover*. After 2 min centrifugation (1027 rcf, at 4 °C) the plate is transferred to a real-time PCR system (e.g., HT7900, Applied Biosystems) and the qPCR reaction is run using the following cycling conditions (specific for our TaqMan assays): 2 min at 50 °C, 15 min at 95 °C, and subsequently 50 cycles of 0:15 min at 94 °C and 1 min at 60 °C each.

3.8 SYBRgreen PCR (for miRNA qPCR Amplification)

1. A mastermix for each individual gene of interest for all samples for quantitative real-time PCR in 25 µl reactions is prepared by mixing 12.5 µl 2× QuantiTect SYBRgreen qPCR Master Mix, 2.5 µl 10× miScript Universal primer mix, 2.5 µl 10× miScript primer assay (for gene of interest, e.g., miR-133b, for details **Note 10**) and 2.5 µl H_2O for each UV-LMD sample/PCR-reaction (volumes are multiplied by the number of samples + controls + 1).
2. 20 µl of mastermix is added to the bottom of a MicroAmp 96-well reaction plate. 5 µl of cDNA (adjust volumes accordingly if cDNA in more or less volume is used) is added to the mastermix and the plate is sealed with an optical adhesive film. After 2 min centrifugation (1027 rcf, at 4 °C) the plate is transferred to a real-time PCR system (i.e., HT7900, Applied Biosystems) and the qPCR reaction is run using the following cycling conditions (specific for miScript assays, Qiagen): 15 min at 95 °C and subsequently 50 cycles, 0:15 min at 94 °C, 0:30 min at 55 °C, 0:30 min at 70 °C, and 0:30 min at 73 °C (fourth segment), followed by a melting curve (0:15 min at 95 °C, 0:15 min at 60 °C, subsequent continuous increase of 1 °C every 0:15 min under the detection of the fluorescence signal up to 95 °C).

3.9 Data Analysis

1. For SYBRgreen PCR, the first step is a melting curve analysis, to ensure PCR-product specificity, as well as correct read-out temperature (fourth segment). Afterwards, data analysis of TaqMan as well as of SyberGreen qPCR are identical.
2. Fluorescence amplification plots are analyzed first without normalization to the internal fluorescence standard dye ROX (carboxy-X-rhodamine, as passive reference dye), to evaluate

absolute fluorescence signals, background noise levels, and possible confounders.

3. TaqMan probe or SYBRgreen fluorescence signals are normalized to the ROX-signals, and the baseline for normalization is set (usually cycles 3–15).
4. It must be ensured that all negative controls did not result in any detectable qPCR signal.
5. The detection threshold is set in the exponential phase of the qPCR amplification plot (illustrated in Fig. 1). To quantify the expression of a respective gene via qPCR for a set of samples, the same threshold value is used for all the samples and for the standards (run in parallel). *Threshold cycle* (C_t) values of each sample as well as slope and Υ-intercept of the standard curve can be read out from the sequence detection software (e.g., SDS2.4, Applied Biosystems).
6. The average cDNA amount per cell in relation to the utilized cDNA standard curve is calculated according to:

$$\text{cDNA amount per cell} = \frac{S^{\left[\left(C_t - \Upsilon_{\text{intercept}}\right)/\text{slope}\right]}}{\text{No}_{\text{cells}} \bullet \text{cDNA fraction}}$$

S corresponds to the serial dilution factor of the standard curve (e.g., 10 for serial dilution in steps of 10), No_{cells} to the number of harvested neurons per sample and cDNA fraction to the fraction of the UV-LMD cDNA sample used as template in the real-time PCR reaction, e.g., 5/55. The unit-magnitude corresponds to the respective standard utilized, which defines the unit at the $\Upsilon_{\text{intercept}}$ (e.g., pg-equivalents of standard cDNA, derived from SN-tissue/cell). For better comparison, expression data can be further normalized to those of control brains (mean controls = 1; compare Figs. 3 and 4d). Alternatively, an absolute standard curve with quantified numbers of RNA or cDNA molecules as templates can be generated, and data are analyzed as described above [61, 62].

3.10 Linear Mixed Effects Model

RT-qPCR data can be further analyzed and corrected for confounding effects, like distinct RINs and ages of human brains, by applying a linear mixed effects model (Figs. 3c and 4c). Our modeling approach assumes a log-linear dependency of RT-qPCR data from age- and RNA-values. Since this is likely to be a simplification, it is mandatory to analyze the goodness-of-fit and applicability of the modeling approach (e.g., R^2 values, proportional change in variance, PCV, and Bayesian information criterion, BIC; for details *see* [46] supplementary material).

Fitting and correction for confounding variables (like age- and RIN-values) and subsequent statistical analysis of adjusted data is carried out on log-transformed expression data, which show a more

symmetric distribution closer to Gaussian. Back-transformation of adjusted data is then done on this assumption of a log-normal distribution. Uncertainties of the regression are considered in the standard errors of means by applying rules of uncertainty and error propagation [63]. From the resulting parameter values (in log-transformed scale) differences in mRNA-levels between control and PD groups are tested for statistical significance by Student's *t*-test (Welch-Test). The general model-based analysis procedure is as follows:

1. Logarithmic transformation of RT-qPCR data.

$$f : \log\aleph(E, \mathrm{Var}) \rightarrow \aleph\left(\mu, \sigma^2\right) \text{ defined by } f\left(\boldsymbol{Y}_{i,j}\right) := \ln \boldsymbol{Y}_{i,j}$$

$\aleph$: normal distribution
$\boldsymbol{Y}_{i,j}$: data vector of measured values for gene i and brain j
E, Var: mean and variance of data (original scale)
μ,σ^2: mean and variance of log-transformed data

2. Fitting of the linear mixed effects model with ASReml-R.

$$\ln \boldsymbol{Y}_{i,j} = \beta_i^{\mathrm{C/PD}} x_j^{\mathrm{C/PD}} + \beta_i^{\mathrm{age}} x_j^{\mathrm{age}} + b_i^{\mathrm{RIN}} x_j^{\mathrm{RIN}} + \left(\beta_i^0 + \gamma_{i,j}^0\right) + \boldsymbol{e}_{i,j}$$

$$b_i^{\mathrm{RIN}} = \beta^{\mathrm{RIN}} + \gamma_i^{\mathrm{RIN}}$$

$\boldsymbol{Y}_{i,j}$: data vector of measured values for gene i and brain j
$\beta_i^{\mathrm{C/PD}}, \beta_i^{\mathrm{age}}, \beta^{\mathrm{RIN}}$: fixed effects for group (control or PD), age and RIN of gene i
$\beta_i^0, \gamma_{i,j}^0$: intercept of gene i and its random contribution for brain j
γ_i^{RIN}: random effect for RIN dependence of gene i
b_i^{RIN}: total RIN dependence of gene i
$\boldsymbol{e}_{i,j}$: residuals (fitted values−mesured values) of each individual gene i and brain j
$x_j^{\mathrm{C/PD}}, x_j^{\mathrm{age}}, x_j^{\mathrm{RIN}}$: group (control, PD), age and RIN value of brain j
(independent variables $y = f(x)$)

3. Adjustment of each RT-qPCR value for confounders (age, RIN).

$$\ln \tilde{\boldsymbol{Y}}_{i,j} = \ln \boldsymbol{Y}_{i,j} + \beta_i^{\mathrm{age}} \Delta x_j^{\mathrm{age}} + \left(\beta^{\mathrm{RIN}} + \gamma_i^{\mathrm{RIN}}\right) \Delta x_j^{\mathrm{RIN}} + \boldsymbol{e}_{i,j}$$

$$\text{with } \Delta x_j^{\mathrm{age}} = \bar{x}_{\mathrm{age}} - x_j^{\mathrm{age}} \text{ and } \Delta x_j^{\mathrm{RIN}} = \bar{x}_{\mathrm{RIN}} - x_j^{\mathrm{RIN}}$$

$\tilde{\boldsymbol{Y}}_{i,j}$: RIN and age adjusted expression values for gene i and brain j

$\boldsymbol{\Upsilon}_{i,j}$: data vector of measured values for gene i and brain j
$\beta_i^{\mathrm{C/PD}}, \beta_i^{\mathrm{age}}, \beta^{\mathrm{RIN}}$: fixed effects for group (control or PD), age and RIN of gene i
$\bar{x}_{\mathrm{age}}$: mean of age values
$\bar{x}_{\mathrm{RIN}}$: mean of RIN values
$x_j^{\mathrm{C/PD}}, x_j^{\mathrm{age}}, x_j^{\mathrm{RIN}}$: group membership (control or PD), age and RIN value of brain j
γ_i^{RIN}: random effect for RIN dependence of gene i
$\boldsymbol{e}_{i,j}$: residuals on single sample level for each gene i and brain j

4. Computation of standard errors for each brain's expression values by applying rules of uncertainty propagation.

$$\theta_{i,j} = \sqrt{\left(\sigma_i^{\mathrm{age}} \Delta x_j^{\mathrm{age}}\right)^2 + \left(\sigma_i^{\mathrm{RIN}} \Delta x_j^{\mathrm{RIN}}\right)^2}$$

with $\Delta x_j^{\mathrm{age}} = \bar{x}_{\mathrm{age}} - x_j^{\mathrm{age}}$ and $\Delta x_j^{\mathrm{RIN}} = \bar{x}_{\mathrm{RIN}} - x_j^{\mathrm{RIN}}$

$\theta_{i,j}$: standard error of adjustment
σ_i^{age}: standard error of age effect β_i^{age} for gene i
σ_i^{RIN}: standard error of RIN effect $\left(\beta^{\mathrm{RIN}} + \gamma_i^{\mathrm{RIN}}\right)$ for gene i

5. Determination of parameters (mean and standard errors) of normal distributions of adjusted values and their adjusted errors.

$$\widetilde{\mu}_{i,k} = \frac{\sum \tilde{\boldsymbol{Y}}_{i,j}}{N_{i,k}}, \quad \widetilde{\sigma}_{i,k}^2 = \mathrm{Var}\left(\tilde{\boldsymbol{Y}}_{i,j}\right) + \frac{\sum \left(\theta_{i,j}\right)^2}{N_{i,k}}$$

for data of brains j belonging to group $k \epsilon \{\mathrm{C}, \mathrm{PD}\}$
$\tilde{\boldsymbol{Y}}_{i,j}$: RIN and age -adjusted expression values for gene i and brain j
$\widetilde{\mu}_{i,k}$: mean of adjusted log-transformed expression values in group k gene i
$\widetilde{\sigma}_{i,k}^2$: variance of adjusted log-transformed expression values in group k for gene i
$N_{i,k}$: number of observations in group k for gene i
Var(): variance of data
$\theta_{i,j}$: standard error of adjustment

6. Test for differences between control and PD groups by applying Student's t-test (Welch-Test).

$$t\text{-test statistic} : t = \frac{\widetilde{\mu}_{i,\mathrm{PD}} - \widetilde{\mu}_{i,\mathrm{C}}}{\sqrt{\widetilde{\mathrm{se}}_{i,\mathrm{PD}}{}^2 + \widetilde{\mathrm{se}}_{i,\mathrm{C}}{}^2}}$$

$$\text{and degrees of freedom : df} = \frac{\left(\widetilde{\text{se}}_{i,\text{PD}}{}^2 + \widetilde{\text{se}}_{i,\text{C}}{}^2\right)^2}{\frac{\widetilde{\text{se}}_{i,\text{PD}}{}^2}{N_{i,\text{PD}}-1} + \frac{\widetilde{\text{se}}_{i,\text{C}}{}^2}{N_{i,\text{C}}-1}}$$

with squared standard error of means:

$$\widetilde{\text{se}}_{i,\text{PD}}{}^2 = \widetilde{\sigma}^2_{i,\text{PD}}/N_{i,\text{PD}} \text{ and } \widetilde{\text{se}}_{i,\text{C}}{}^2 = \widetilde{\sigma}^2_{i,\text{C}}/N_{i,\text{C}}$$

$\widetilde{\mu}_{i,\text{C/PD}}$: mean of adjusted log-transformed expression values for gene i from C or PD
$\widetilde{\sigma}^2_{i,\text{C/PD}}$: variance of adjusted log-transformed expression values for gene i in C or PD
$N_{i,\text{C/PD}}$: number of observations for gene i in C or PD group

7. Back-transformation from log-transformed to original scale by the computation of mean values and variances from parameter values of normal distributions in the transformed space.

$$\tilde{E}_{i,k} = e^{\widetilde{\mu}_{i,k}+\widetilde{\sigma}^2_{i,k}/2}, \qquad \widetilde{\text{Var}}_{i,k} = \left(e^{\widetilde{\sigma}^2_{i,k}} - 1\right) e^{2\widetilde{\mu}_{i,k}+\widetilde{\sigma}^2_{i,k}}$$

with $k\epsilon\{\text{C, PD}\}$
$\tilde{E}_{i,k}$: mean of adjusted data in original scale (pg cDNA/cell)
$\widetilde{\mu}_{i,k}$: mean of adjusted log-transformed expression values in group k for gene i
$\widetilde{\sigma}^2_{i,k}$: variance of adjusted log-transformed expression values in group k for gene i
$\widetilde{\text{Var}}_{i,k}$: variance of adjusted data in original scale

8. To assess goodness-of-fit, R^2 values as well as *proportional change in variance* (PCV) are determined at the brain level, as suggested in [58, 64].

$$R^2_i = 1 - \frac{\text{MSPE}_i}{\text{MSPE}_{0,i}} \text{ with mean squared prediction error MSPE}_i$$
$$= \frac{\text{Var}(\boldsymbol{e}_i)}{n_i} + \text{Var}\left(\boldsymbol{\gamma}^0_{\boldsymbol{i}}\right)$$

$\text{MSPE}_{0,i}$: MSPE of a reference model, e.g., the empty model of gene i
Var ($\boldsymbol{e}_i$): variance of residuals for gene i
n_i: number of samples from each brain for gene i (n_i=10)
$\text{Var}\left(\boldsymbol{\gamma}^0_{\boldsymbol{i}}\right)$: variance of random effects for gene i

and

$$R^2_{\text{marg},i} = \frac{\sigma_f^2}{\sigma_f^2 + \text{Var}\left(\gamma_i^0\right) + \text{Var}(\boldsymbol{e_i})}, \quad R^2_{\text{cond},i} = \frac{\sigma_f^2 + \text{Var}\left(\gamma_i^0\right)}{\sigma_f^2 + \text{Var}\left(\gamma_i^0\right) + \text{Var}(\boldsymbol{e_i})}$$

$R^2_{\text{marg},i}$: marginal R^2, variance explained by fixed effects only.
$R^2_{\text{cond},i}$: conditional R^2, variance explained by fixed and random effects.
$\sigma^2_{f,i}$: variance calculated from the fixed effect components of the mixed effects model.

and PCV:

$$\text{PCV}_i = 1 - \frac{\text{Var}\left(\gamma_i^0\right)}{\text{Var}\left(\gamma^0_{\text{ref},i}\right)}$$

$\text{Var}\left(\gamma^0_{\text{ref},i}\right)$: variance of random effects of reference model, e.g., the empty model.

9. In addition, a relative adjustment error can be computed as

$$\varepsilon_i = \frac{\sum \theta_{i,j} / \boldsymbol{Y}_{i,j}}{N_i}$$

$\theta_{i,j}$: standard error of adjustment
$\boldsymbol{\Upsilon}_{i,j}$: data vector of mesured values for gene i and brain j
$\boldsymbol{Y}_{i,j}$: data vector of measured values for gene i and brain j
N_i : number of observations for gene i

The different R^2, PCV, and error values allow an evaluation of the model fit and the adjustment quality. R^2 coefficients could be regarded as a measure of variance explained by different aspects of the model, with values closer to one meaning better explanatory power. R_i^2 is closest to the *coefficient of determination* from standard linear regression, but should, according to [58], be supplemented by so-called *marginal (marg)* and *conditional (cond)* R^2 values, if a mixed effects model is evaluated. Since we are interested in the influence of fixed effects, $R^2_{\text{marg},i}$ is of special importance. The conditional coefficient $R^2_{\text{cond},i}$ additionally accounts for the influence of random effects. The difference between $R^2_{\text{cond},i}$ and $R^2_{\text{marg},i}$ gives insight to which extent inter-individual differences that are not explained by fixed effects are captured by the model through random effects. High PCV values express how well certain fixed effects are able to reduce the contribution of random effects to the explanatory power of the model. The relative adjustment error ε_i gives the mean of the summed standard errors from adjustment in relation to the expression value for each gene i. Small values indicate low uncertainties by the adjustment procedure.

4 Notes

1. Ribonuclease contamination is a major concern for successful cDNA synthesis of single-laser microdissected cells or small cell pools. The ubiquitous RNase A is a highly stable and active ribonuclease, which is present on human skin as well as in the specimens, and can easily contaminate any lab environment. Thus, creating and maintaining an RNase-free work environment and RNase-free solutions is essential for performing successful reverse transcriptase reactions. Therefore, we strongly recommend: Always wear gloves when handling chemicals and sections/samples containing RNA. Change gloves frequently especially after touching potential sources of RNase contamination such as doorknobs, pens, pencils, and human skin. Always use certified RNase-free tubes, pipette tips and chemicals *for all steps* involved in the experiments (e.g., ethanol/staining solutions and jars for the preparation of tissue sections for UV-LMD). Keep chemicals tightly sealed. Keep all the tubes containing RNA tightly sealed during the incubation steps. Treat UV-LMD (membrane-) slides for 20 min with UVC-light (e.g., in a sterile hood). Heat sterilize all metal (forceps, spatulas, LMD cap holder, LMD slide holder), glassware and any other equipment that gets in contact with slides or reaction tubes during UV-LMD experiments at 220 °C overnight. Clean pipettes, benches, and all other equipment that cannot be heat sterilized with RNase decontamination solutions, e.g., RNase-ExitusPlus (AppliChem) and/or RNaseZapWipes (Ambion). Clean the cryostat additionally with isopropanol (Isopropanol, Sigma-Aldrich) to wipe off RNaseZap.
2. Low retention filter tips should be used for all pipetting steps.
3. We strongly recommend using chemicals from the same stocks and lots for all experiments of a study.
4. To avoid any RNase or DNA contamination, we recommend preparing the *Cap-Mix* under a sterile fume hood.
5. As RNA in human midbrain tissue is likely already partially degraded, we recommend using qPCR amplicon sizes below 80 bp when working with human tissue.
6. To reuse the specimen for several experiments, brains are fixed on cork discs with tissue freezing medium. These cork discs can be frozen quickly with a drop of water on the specimen holder of the cryostat and easily be removed after the experiment and stored again at −80 °C.
7. Tissue chippings of the cryosectioning procedure are used to assess overall RNA quality and tissue pH of each specimen.

Transfer chippings into a liquid nitrogen precooled Falcon tube with a cold forceps and store at −80 °C until further usage (e.g., pH-determination, or RNA extraction and small RNA, miRNA and RNA integrity number evaluation).

8. PEN-membrane slides with tissue sections can be stored in 50 ml Falcon tubes at −80 °C and reused for later experiments. To ensure that the slides stay dry, silica gel is added to the Falcon tube (Fig. 1). A small sieve is used to separate the silica gel from the slide. For reuse, the slides are removed from −80 °C and allowed to equilibrate at −20 °C (20 min), +4 °C (20 min), and finally at room temperature (20 min) before usage.
9. Our mild lysis protocols are optimized for single UV-laser microdissected cells or small pools of individual cells from ethanol-fixed tissue sections. Please note that they are neither suited for lysis of larger microdissected tissue samples, nor for lysis of single cells from PFA-fixed tissue sections.
10. Assay ID numbers for TaqMan assays (Life Technologies) are: SNCA: Hs00240906_m1, TH: Hs00165941_m1, DAT: Hs00997374_m1, VMAT2: Hs00996839_m1, NURR1: Hs00428691_m1, LDH-2: Mm00493146_m1, PITX3: custom assay, forward primer: GCACGGCTGCAAGGG, reverse primer: GGCTTCAGGTTCGTAGTCTTGAT; probe: FAM-ACCCTTCCTTGCCCAACTG-NFQ. Assay ID number for miR-133b SYBRGreen assay (Qiagen) is: MS00007385.

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Chapter 20

Laser-Capture Microdissection for Layer-Specific Analysis of Enteric Ganglia

Corinna Rosenbaum, Martina Böttner, Thilo Wedel, and Marco Metzger

Abstract

The enteric nervous system (ENS) is the division of the autonomic nervous system that innervates the gastrointestinal (GI) tract and controls central intestinal functions such as peristalsis and fluid movement. Enteric nerve cell bodies (neurons and glia) are predominantly organized in ganglionated networks that are present along the entire length of the GI tract in multiple tissue layers. Most cell bodies are organized in the myenteric plexus allocated between the longitudinal and the circular muscle layers or in the submucosal plexus between muscle tissue and mucosa. The site-specific characteristics of these enteric nerve cells have traditionally been analyzed via imaging techniques. Laser-capture microdissection (LCM) offers the prospect of site-specifically analyzing the gene expression profiles of these different subpopulations. This protocol addresses critical aspects of handling intestinal tissue for ENS dissection, such as the optimal quick-staining procedure, suitable laser settings, and limits of tissue material required to successfully dissect and analyze tissue layers for gene expression.

Key words Laser-capture microdissection, Enteric nervous system, Enteric neuronal and glial cells, Enteric plexus

1 Introduction

The enteric nervous system (ENS) is the largest division of the autonomic nervous system and innervates the entire gastrointestinal (GI) tract. Its function is to orchestrate the actions of smooth muscle cells, absorptive and secretory epithelial cells, and blood vessels into an organized digestive behavior. The major proportion of enteric nerve cells is allocated in the muscular layer between the longitudinal and the circular muscle, forming the ganglionated myenteric plexus. Its main function is the control of intestinal motility. Other ganglionated networks extend throughout the submucosa of the intestinal wall, mediating mainly secretory and absorptive functions [1]. Additionally, nerve cell bodies and nerve fibers span the mucosa [1]. Beside this anatomical diversity, the ENS is also highly heterogeneous on a cellular and functional level,

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comprising different subtypes of neurons and glial cells with specialized functions depending on their neurochemical characteristics [2].

The site-specific complexity of the ENS has been studied mainly via imaging techniques, including immunohistochemistry [3], the use of indicator dyes [4, 5], or reporter strains. Since most imaging techniques involve either serial sectioning of gut tissue samples or optical sectioning by means of confocal and two-photon microscopy, an assessment of the tissue architecture is an intrinsic characteristic of these techniques. For gene expression profiling, however, a site-specific discrimination of the different plexus structures is not possible when intestinal tissue homogenates are used [6]. One approach to analyzing gene expression in layer-specific plexus structures relies on mechanical dissection of tissue layers under stereomicroscopic control prior to RNA isolation, mostly to isolate longitudinal muscle-myenteric plexus (LMMP) segments [7, 8], but also to collect the submucosa [7, 9] or the mucosa [9, 10]. With laser-aided microdissection it became possible to accurately isolate individual ganglia for subsequent gene expression analysis of receptor [10–14], neurotransmitter [15], and growth factor [16–18] expression. Laser-capture microdissection (LCM) has not only been employed to isolate individual myenteric ganglia, but to precisely separate individual intestinal tissue layers, such as the longitudinal from the circular muscle layer [10, 11] or the submucosal layer from the mucosa [19]. The most relevant ENS genes that have been analyzed in microdissected samples include overall neuronal markers (NGFR, RET, PGP 9.5) [11, 12, 14, 16], neuronal subtype markers (NOS1, CHAT, TH, SERT, 5HT-4R, NPY, sodium channels) [10, 14, 15], and glial markers (S100B, GFAP) [14, 16, 19]. These studies have provided valuable knowledge on the site-specific heterogeneity of the ENS and alterations thereof mediated by aging [14] or diseases [10, 19].

When handling intestinal tissue to dissect ENS components, considerations have to be made on (1) the optimal quick-staining procedure to visualize the structures of interest, (2) the optimal laser energy settings to dissect different tissue layers from a single cryosection, (3) prevention of cryosection detachment once several adjacent segments have been cut out, (4) differing RNA qualities to be expected when isolating different tissue types such as dense muscular tissue or enzyme-rich mucosa. These aspects will be addressed in the following protocol.

2 Materials

2.1 Reagents and Consumables

1. RNase-free conical tubes (50 ml).
2. RNase-free filter tips.

3. Water, PCR grade.
4. Ethanol.
5. Cresyl violet staining solution: 1% (w/v) Cresyl violet acetate (Sigma-Aldrich, Munich, Germany) in 50% Ethanol, filtered before use.
6. Filtering paper.
7. PEN-membrane coated microscopy slides, NF 1.0 (Carl Zeiss, Jena, Germany).
8. Capture device AdhesiveCaps opaque (Carl Zeiss).
9. Lysis buffer: RLT lysis buffer from RNeasy Micro Kit, supplemented with 1% (v/v) β-Mercaptoethanol (Sigma-Aldrich).
10. RNeasy Micro Kit for RNA isolation (Qiagen, Hilden, Germany).

2.2 Tools and Instruments

1. Cabinet for UV light exposure.
2. Glass staining jars with covers.
3. Glass funnel.
4. Forceps.
5. Cryotome.
6. PALM MicroBeam System with PALM RoboStage (Carl Zeiss) integrated in an inverse light microscope (e.g., Axio Observer D1, Carl Zeiss).

3 Methods

Take measures to avoid exposure to RNases, including wearing gloves and using RNase-free solutions, consumables, and decontaminated glassware.

3.1 Sample Preparation

1. Prepare polyethylene naphtalate (PEN)-membrane coated microscopy slides by exposure to UV-light for 30 min to counteract the membrane's hydrophobic character and destroy potentially contaminating nucleic acids.
2. Mount cryosections (10–16 μm) of unfixed cryo-embedded intestinal full-thickness sections on PEN-membranes (*see* **Note 1**). Air-dry for 10 min and store at −80 °C in sealed RNase-free conical tubes.

3.2 Quick-Staining of Cryosections

1. Bake glass jars and covers for the different staining solutions at 180 °C for RNase decontamination.
2. Fill jars with RNase-free water, 70% ethanol, 100% ethanol and cresyl violet staining solution. Store them closed and use at 4 °C (*see* **Note 2**).

3. Thaw membrane slide in a sealed RNase-free conical tube to avoid excess condensation of moisture.
4. Handle membrane slide with forceps. Dip slide in RNase-free water to remove excess embedding compound.
5. Place the slide in ice-cold 70% ethanol for 2 min for fixation.
6. Place the slide in cresyl violet staining solution for 30 s.
7. Let excess staining solution drop on an absorbent surface (e.g., paper towel).
8. Dip the slide in 70% ethanol.
9. Dip the slide in 100% ethanol.
10. Air-dry membrane slide quickly under fume hood (*see* **Note 3**).

3.3 Laser Microdissection

1. Place the slide in a slide holding frame on the computer-controlled motorized PALM RoboStage and position stage with the PALM Robo-Software.
2. Visualize the tissue specimen in a suitable magnification.
3. Use the software-implemented "Cut Laser Adjustment Wizard" to adjust the laser energy and laser focus by following the instructions given by the software (*see* **Note 4**). Cut laser energy settings of 54 ± 2 and cut laser focus settings of 73 ± 2 in the PALM Robo software were found suitable to excise myenteric ganglia out of 12 μm cryosections of rat intestinal tissue at 20× magnification (*see* **Note 5**). Cut laser energy settings of 63 ± 2 and cut laser focus settings of 85 ± 2 were found suitable to excise myenteric ganglia out of 16 μm cryosections of human colon tissue at 10× magnification (*see* **Note 6**).
4. Place the capture device, e.g., AdhesiveCap opaque in the suitable collection device mounted on the PALM CapMover, for instance a 500 μl AdhesiveCap in a Tube collector 500. Wear gloves when handling the capture device (*see* **Note 7**).
5. Open the Capture Device Dialog in the PALM Robo-Software and select the collector device you are using. Align the AdhesiveCap over your sample.
6. Select a cutting mode, it is advised to use the RoboLPC mode (*see* **Note 8**).
7. Select a drawing mode, it is advised to use the freehand tool. Select the tissue region of interest (ROI) for dissection (*see* Fig. 1). If LCM is used to separate myenteric from submucosal regions, spacious ROIs can be drawn (*see* Fig. 2).
8. Collect multiple tissue areas to ensure sufficient RNA yield. For precise excision of myenteric ganglia, a number of ganglia ranging from 50 ganglia [14] up to 150–200 ganglia (corresponding to 4 mm^2) [16] have been reported per sample

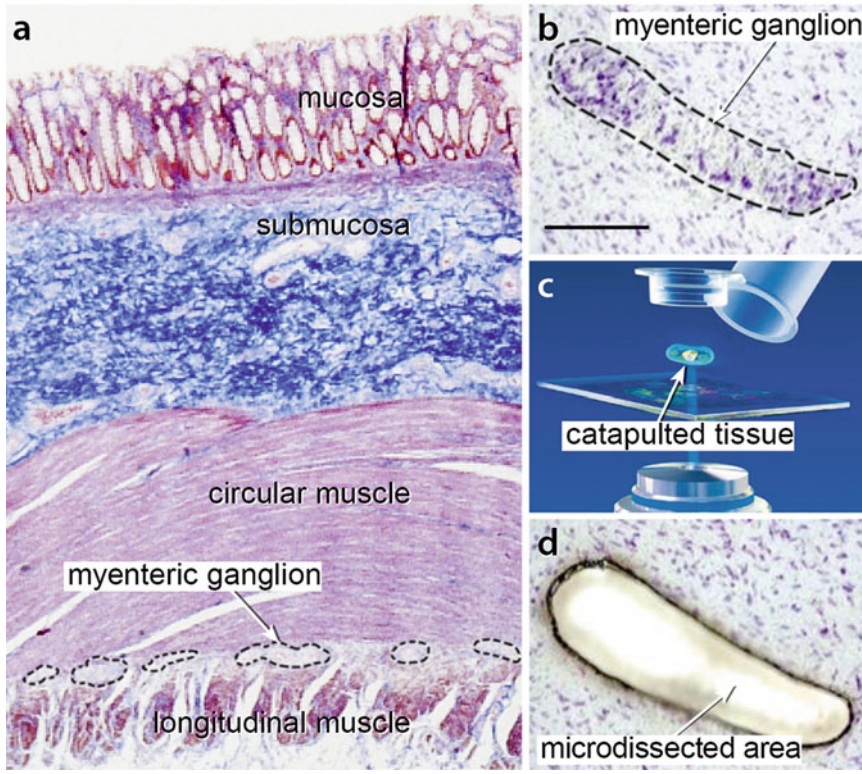

Fig. 1 Isolation of myenteric ganglia from full-thickness sections of human colon by laser-capture microdissection (LCM). (**a**) Full-thickness section of human colon displaying the mucosa, submucosa, and muscular layers containing myenteric ganglia (dotted lines; Azan staining on paraffin section). (**b**) Myenteric ganglion (dotted line) between circular and longitudinal muscle layer identified by purple-colored prominent neuronal somata (Cresyl violet staining on cryosection). Scale = 100 μm (**c**) Schematic illustration of LCM showing catapulting of the microdissected area from the tissue section into the cap of the reaction tube (with kind permission from Zeiss). (**d**) Same specimen as in (**b**) with microdissected area corresponding to the isolated myenteric ganglion

(*see* **Note 9**). For less accurate tissue layer separation, tissue areas of 1.5–2 mm^2 were found suitable for myenteric plexus regions, 2.5 mm^2 for submucosal plexus regions, and >2.5 mm^2 for mucosal segments (*see* **Note 10**). Keep in mind that the expression levels of ENS genes correlate with the number of ENS cell bodies in the respective tissue layer: expression levels are generally highest in the myenteric region and lowest in the mucosa (*see* Fig. 2).

9. Remove the collection device and add 350 μl cell lysis buffer. Place AdhesiveCap upside-down and incubate lysis buffer for 30 min at room temperature. Quickly vortex and either store lysate at −80 °C or directly proceed with RNA isolation using a suitable RNA isolation kit, e.g., RNeasy Micro Kit (*see* **Note 11**).

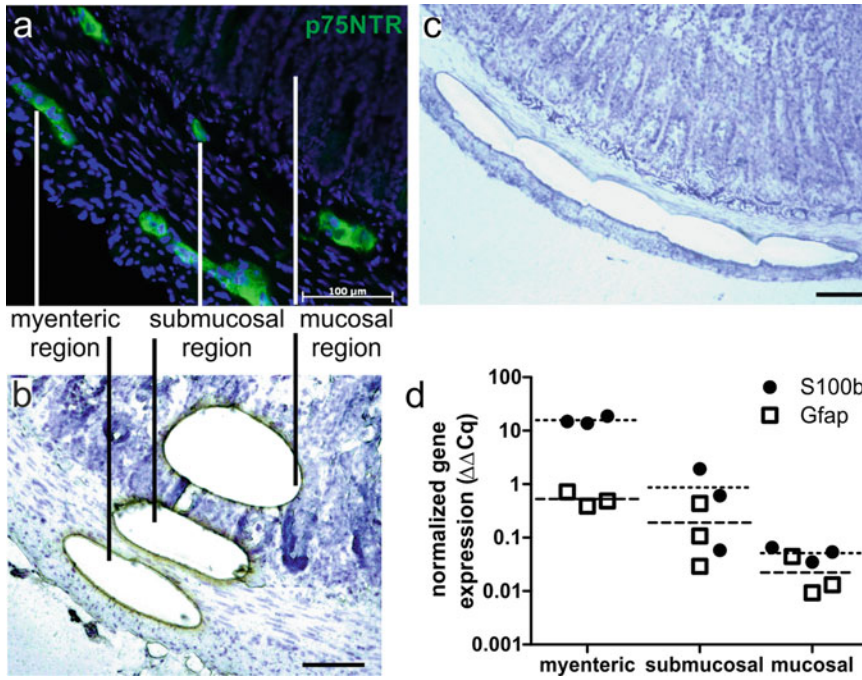

Fig. 2 Isolation of myenteric, submucosal and mucosal regions from full-thickness sections of rat small intestine by LCM. (**a**) p75 neurotrophin receptor (p75NTR) immunohistochemical staining of full-thickness section of rat small intestine indicating myenteric and submucosal plexus structures. (**b**) Cresyl violet-stained tissue section showing exemplary myenteric, submucosal, and mucosal tissue segments cut and catapulted for mRNA transcript level analysis. (**c**) Cresyl violet-stained section showing the sequential excision of myenteric region segments. (**d**) Absolute normalized gene expression (ΔΔCq) of the glial genes S100b (circles) and Gfap (squares) in myenteric, submucosal, and mucosal segments excised from a rat duodenal tissue section plotted on a logarithmic scale. Expression levels for both glial genes decrease considerably along the myenteric-mucosal axis. Scale = 100 µm. Figure adapted from [19]

4 Notes

1. The protocol presented here is optimized for unfixed, cryo-embedded tissue. While processing the tissue for cryo-embedding, it is crucial to prevent RNA degradation, therefore the tissue must be cooled, handled quickly and with RNase-free equipment. If RNA should be isolated from formalin-fixed and paraffin-embedded (FFPE) tissue, an alternative RNA kit has to be employed, e.g., RNeasy FFPE kit (Qiagen). Keep in mind that formalin fixation causes cross-linking of proteins and nucleic acids and thereby reduces RNA integrity.
2. Using cresyl violet as staining solution is recommended since it is a quick, non-aqueous staining procedure that limits the activity of RNases. The staining is sufficient to identify

myenteric ganglia in human colon samples by their large neuronal nuclei and purple neuronal cytoplasm (*see* Fig. 1). Alternative histological dyes have been used to quick-staining enteric ganglia, e.g., hematoxylin [14] or hematoxylin-equivalent staining solutions (e.g., Histogene LCM staining kit (Arcturus, Mountain View, USA) [12, 13] as well as toluidine blue [16]. Keep the time of incubation as short as possible, especially in aqueous solutions. Staining solutions can be reused several times but should be exchanged regularly.

3. The membrane slide must be completely dry for LCM. Remaining moisture will impede catapulting and lead to a heating of the tissue specimen.
4. Laser energy and laser focus settings depend on the magnification used and must be adjusted for every objective.
5. Thicker sections yield more isolated tissue, but require higher laser power to excise the ROI. Isolated ganglia from sections which are too thick might not be catapulted in the cap of the collection tube. In addition, the laser settings might depend on the age of the laser.
6. The different intestinal tissue layers (muscle and mucosa) may require slightly different laser energy settings. It was found that mucosal segments required reduced laser energy and laser focus settings than muscular layer. When excising the submucosal plexus region, it is advised to increase the cutting iteration to two cycles, possibly with a slightly altered cut laser focus setting (z-focus delta), to ensure the complete cutting of both the adjacent muscular and mucosal tissues. When excising myenteric ganglia, make sure to cut exactly along the border between neural and muscular tissue. When cutting too generously, the ganglionic tissue will be contaminated with muscle tissue, when cutting only the area of the myenteric ganglia, tissue loss will occur due to the laser burning the ROI (see also **Note 9**).
7. AdhesiveCaps opaque are PCR tubes with caps filled with an adhesive material that allows dry sample collection. Alternatively, standard tubes can be used with lids lined with an RNA-stabilizing agent (for example RNAlater®, Qiagen) [14] or with RNA lysis buffer. When choosing a liquid collection method, PCR tubes have to be removed very carefully from the collection device, so that no sample-containing liquid is spilled. Some protocols suggest using mineral oil to line the cap; however, we did not find mineral oil suitable for sample collection.
8. The cutting mode RoboLPC sets the position for the catapulting impulse along the cutting line, whereas the Center RoboLPC function sets the catapulting impulse in the center

of the excised area. The latter will facilitate the catapulting, but at the cost of burning the central ROI.

9. To validate that only ganglionated structures and no surrounding muscle tissue have been excised, gene expression of smooth muscle markers such as tropomyosin (TPM) can be analyzed [16]. Additionally, muscle tissue adjacent to the ganglia can be excised as control tissue to validate the significantly reduced expression levels of ENS genes [14].
10. When excising spacious and directly adjacent ROIs, cryosections have been observed to roll up due to lack of supporting PEN membrane. Detachment of cryosections makes cutting and catapulting in these areas not feasible. It is advised to periodically leave a segment of the section uncut to avoid detachment.
11. RNA quality and quantity can be tested prior to cDNA synthesis with an automated electrophoresis system, e.g., Bioanalyzer (Agilent) or Experion (BioRad). Due to the low RNA yield from laser-dissected samples, it might be reasonable to assess RNA parameters in exemplary samples only. RNA concentrations of 1–10 ng/μl with RNA quality indicator (RQI) values greater 7 can be expected. If it is not possible to measure RNA levels in the samples, it is recommended to use the total RNA volume for cDNA synthesis.

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Chapter 21

A Laser Microdissection–Liquid Chromatography–Tandem Mass Spectrometry Workflow for Post-mortem Analysis of Brain Tissue

David C. Hondius, Jeroen J. M. Hoozemans, Annemieke J. M. Rozemuller, Ka Wan Li, and August B. Smit

Abstract

Improved speed and sensitivity of mass spectrometry allow the simultaneous quantification of high numbers of proteins from increasingly smaller quantities of tissue sample. Quantitative data of the proteome is highly valuable for providing unbiased information on, for example, protein expression changes related to disease or identifying related biomarkers. In brain diseases the affected area can be small and pathogenic events can be related to a specific cell type in an otherwise heterogeneous tissue type. An emerging approach dedicated to analyzing this type of samples is laser micro-dissection (LMD) combined with LC-MS/MS into a single workflow. In this chapter, we describe different options for isolating tissue suitable for LC-MS/MS analysis.

Key words Laser microdissection, Laser capture, Post-mortem tissue, Human brain, Proteomics, Mass spectrometry, Immunohistochemistry, Single cell, Protein aggregates, Inclusion bodies

1 Introduction

Improved speed and sensitivity of mass spectrometry allow the simultaneous quantification of high numbers of proteins from increasingly smaller quantities of tissue sample. Quantitative data of the proteome is highly valuable for providing unbiased information on, for example, protein expression changes related to disease or identifying related biomarkers.

In brain diseases the affected area can be small and pathogenic events can be related to a specific cell type in an otherwise heterogeneous tissue type. An emerging approach dedicated to analyzing this type of samples is laser micro-dissection (LMD) combined with LC-MS/MS into a single workflow. LMD enables isolation of small pieces of tissue, down to cells or even cellular inclusions, with high precision for mass spectrometric analysis [1].

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In this chapter, we describe different options for isolating tissue suitable for LC-MS/MS analysis. Methods are divided into four variants. Protocol A describes tissue isolation from a specific brain area exemplified by the isolation of CA1 and subiculum from hippocampus, as published previously [2]. Protocol B describes tissue isolation of areas with high pathological burden, as visualized with a fast immunohistochemical (IHC) protocol. Protocol C describes isolation of inclusion bodies from separate cells using histochemical (HC) staining as published previously [3]. Protocol D describes isolation of separate cells that are positive for a specific marker using a fast IHC protocol.

The methods described here include tissue preparation, different visualization methods, know-how on the LMD procedure, and further processing for MS analysis. Generally, we have made use of a Leica LMD 6500 system and in some cases of a Zeiss PALM microbeam system. We found that both systems deliver outstanding results. Depending on the tissue and the research question, the methods might require some adjustment. This chapter aims at providing the protocols to apply the LMD - LC-MS/MS workflow successfully.

2 Materials

Prepare all the solutions using ultrapure (sterile) water. Prepare and store all reagents at room temperature (unless indicated otherwise). It is important to work as clean as possible considering that the downstream MS analysis is very sensitive and will detect impurities that were introduced while preparing the tissue samples.

2.1 Preparation of Tissue for LMD Including (Immuno-) Histochemical Staining ((I)HC)

1. Snap frozen, post-mortem, human brain tissue with short (<12 h) post-mortem delay (*see* **Note 1**).
2. Cryostat (Leica).
3. Polyethylene naphthalate (PEN)-foil membrane slides (Leica).
4. Ethanol 100%.
5. Ultrapure sterile H_2O.
6. Toluidine blue (Sigma-Aldrich) 1% (w/v) in sterile H_2O.
7. Hair dryer.
8. For additional protocol-specific requirements, *see* Subheading 2.2 (for protocols B and D) and Subheading 2.3 (for protocol C).

2.2 Preparation of Tissue for LMD Using an Immunostaining (IHC) (Protocols B and D only)

1. Sterile PBS, (pH 7.4): NaCl 8.2 g, $Na_2HPO_4 \cdot 12H_2O$ 3.1 g, $NaH_2PO_4 \cdot 2H_2O$ 0.3 g in 1000 mL.
2. Primary antibody (anti beta-amyloid (clone IC16, Kind gift of Prof. Carsten Korth) and anti phospho-tau (clone AT8, Thermo scientific) for protocol B and D, respectively).

3. HRP-labeled secondary antibody (protocols B and C only) Goat anti-mouse-HRP (DAKO).
4. 3,3′-diaminobenzidine (DAB) in chromogen solution (DAKO).

2.3 Preparation of Tissue for LMD, Applying a Histochemical (HC) Staining Using Eosin (Protocol C only)

1. Eosin staining solution: 50% (v/v) ethanol 0.2% (w/v) eosin and 0.04% (v/v) acetic acid.

2.4 Laser Microdissection

1. Laser dissection microscope (here a Leica LMD6500 system was mostly used. Some experiments have also been performed using a Zeiss PALM MicroBeam system).
2. 0.5 mL cap (Greiner Bio-one) or adhesive caps (Zeiss). Adhesive caps are obligatory when using a Zeiss PALM system but are useful in certain cases for the Leica system as well (*see* **Note 2**).
3. Pierce™ Lane Marker Reducing Sample Buffer 5× (Thermo scientific) (*see* **Note 2**).
4. M-PER lysis buffer (Thermo scientific).

2.5 SDS-PAGE

1. Centrifuge (Eppendorf, 5415D or comparable).
2. Pierce™ Lane Marker Reducing Sample Buffer 5× (Thermo scientific).
3. M-PER lysis buffer (Thermo scientific).
4. Water bath at 95 °C.
5. Prestained protein ladder (Bio-Rad).
6. Gel fixation solution: 50% Ethanol (v/v), 3% Phosphoric Acid (from 85% stock) in H_2O.
7. Colloidal Coomassie blue: 34% (v/v) Methanol, 3% (v/v) Phosphoric Acid (from 85% stock) (Sigma Aldrich), 150 g Ammonium Sulphate, 1 g Coomassie brilliant blue G-250 (Thermo Scientific) in H_2O.

2.6 SDS PAGE for Larger Isolated Areas (Multiple Fractions) (Protocols A and B)

1. NuPAGE® 4–12% Bis-Tris acrylamide gel (Invitrogen).
2. MOPS running buffer (Invitrogen).

2.7 SDS Page for Single Cell/ Inclusion Body Analysis (Single Fraction) (Protocols C and D)

1. Acrylamide/Bis Solution, 19:1 (Bio-rad).
2. Tris–HCl 1.5 M, adjusted to pH 8.8 using HCl.
3. 10% w/v SDS.
4. Ultra pure (sterile) H_2O.
5. Ammonium persulfate: 10% (w/v) solution in H_2O.
6. TEMED (Bio-rad).
7. Mini-PROTEAN® 3 Cell (Bio-rad).
8. Glass plates with 1 mm spacers.
9. 10-well comb.
10. 10× Tris/Glycine/SDS (Bio-rad).

2.8 In Gel Digestion

1. Ammonium bicarbonate (100 mM): 0.78 g NH_3HCO_3 fill up to 100 mL with deionized water.
2. Acetonitrile (HPLC grade).
3. Capillary gel loading pipet tips (VWR).
4. Trypsin/Lys-C Mix, Mass spec Grade (20 μg per vial from Promega).

2.9 Mass Spectrometry Analysis

1. A capillary HPLC system with autosampler.
2. HPLC solvents A: 94.9% deionized water/5% acetonitrile/0.1% formic acid, and B: 94.9% acetonitrile/5% deionized water/0.1% formic acid.
3. An electrospray mass spectrometer. Mass spectrometers based on the use of either Orbitrap or quadrupole-time of flight mass analyzer are preferred. We describe the use of the Sciex Triple TOF 5600 system as an example.

3 Methods

Carry out all the procedures at room temperature unless otherwise specified.

3.1 Preparation of the Slides for Microdissection

1. Prepare PEN-foil slides by placing them in UV light for 30 min according to the manufacturer's instructions.
2. Tissue sections are cut at the desired thickness in a cryostat at a temperature of −16 °C to −18 °C and applied on PEN-foil slides (*see* **Note 3**). For experiments A and B (larger areas of interest) typically the thickness used is between 10 and 50 μm depending on the tissue and the type of laser dissection microscope (*see* **Note 4**). For experiments C and D (separate cells/inclusion bodies) the thickness used is typically 10 μm or less (*see* **Note 5**).

3. Let the tissue sections air-dry for at least 10 min.
4. Fix the sections in 100% ethanol for 1 min (*see* **Notes 6** and 7).
5. Air-dry for 10 min (or dry using a hair dryer, set to cool air, for 1 min).
6. Proceed with Subheading 3.2 (protocol A), Subheading 3.3 (protocols B and D), or Subheading 3.4 (protocol D).

3.2 Preparing Section with Toluidine Blue Staining (Protocol A)

1. Briefly wet the sections with sterile ultra-pure H_2O by pipetting it on the sections.
2. Remove excess H_2O by tilting the slide.
3. Apply a few drops of toluidine (1% v/v in H_2O) onto the tissue in such a manner that all the tissue is covered. Incubate for 1 min.
4. Wash the slides twice for 30 s in H_2O. (Use two containers with a volume of 250 mL or more to do the washing).
5. Wash the slides in ethanol 100% three times 30 s.
6. Air-dry for 10 min (or dry using a hair dryer, set to cool air, for 1 min).
7. Store at room temperature (RT) till further use (within 1 week) (*see* **Note 8**).
   a. A typical image obtained using this protocol is shown in Fig. 1a.
8. Proceed to Subheading 3.5.

3.3 Fast IHC Staining (Protocols B and D)

1. Prepare all antibody dilutions in sterile PBS (*see* **Note 9**). Although depending on the section size, approximately 100 µL of diluted antibody is sufficient to completely cover the tissue section.
2. Prepare DAB solution according to the manufacturer's protocol.
3. Briefly wet the sections using sterile PBS (pH 7.4).
4. Remove excess fluid by tilting the slide.
5. Apply the primary antibody. Make sure the entire tissue section is covered. This can be done by distributing the liquid with the back of a pipet tip. Make sure not to touch the tissue or damage the membrane. Incubate for 20 min.
6. Wash three times 30 s in sterile PBS (pH 7.4) (Use three containers with a volume of 250 mL or more to do the washing).
7. Apply the secondary HRP-labeled antibody. Use the highest dilution mentioned in the accompanying datasheet (DAKO) that is suitable for cytochemistry. Usually, 1:100 is used but this depends on the antibody. Incubate for 20 min.

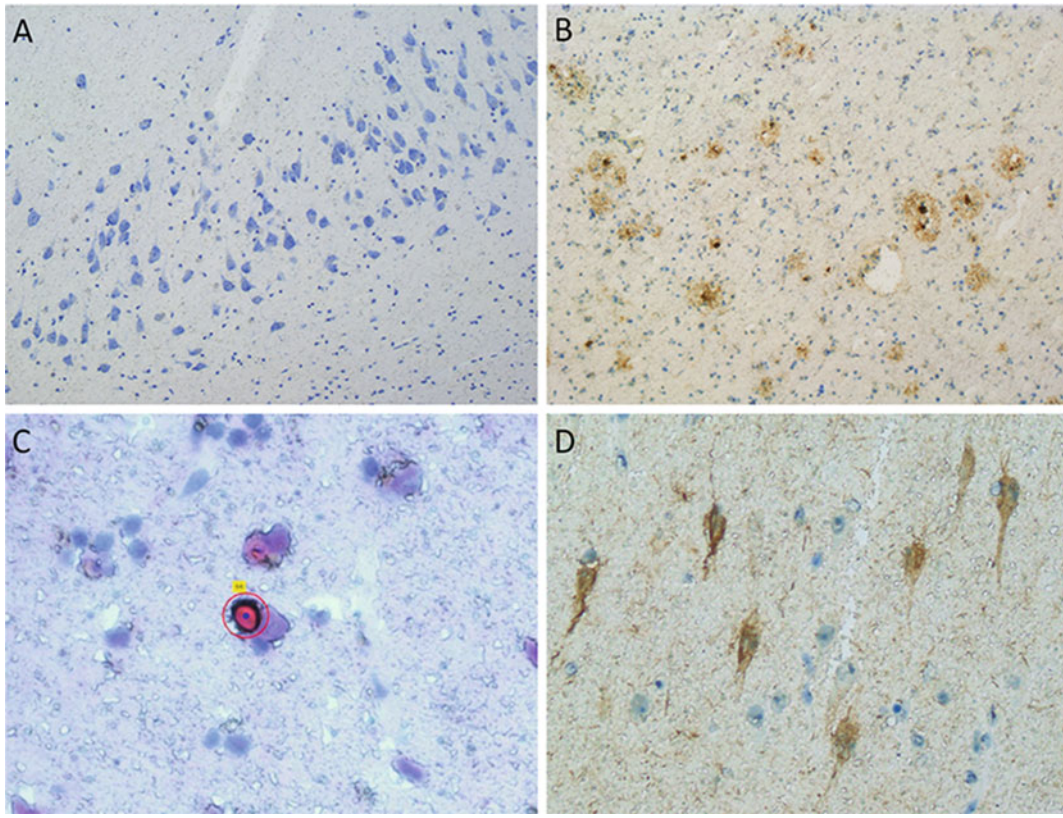

Fig. 1 Typical images obtained using the different (I)HC protocols. (**a**) Toluidine blue staining of human hippocampus. Shown is the CA1 region of the hippocampus. (**b**) Amyloid beta plaques identified using IHC. Immunodetection and visualization of beta-amyloid in plaques using DAB (brown). Toluidine blue was used as a counterstain (blue). (**c**) Inclusion body stained using eosin. (**d**) Cells displaying immunoreactivity for phospho-tau visualized using DAB (brown), toluidine blue was used as a counterstain (blue)

8. Wash three times 30 s in sterile PBS (pH 7.4).
9. Apply the DAB solution to the slides. Incubate for 5 min in the dark.
10. Thoroughly wash repeatedly with ultrapure H_2O.
11. Incubate with 1% (w/v) toluidine blue in H_2O for 1 min to implement a counterstain.
12. Wash the slides twice for 30 s in H_2O.
13. Wash the slides in ethanol 100% three times for 30 s.
14. Air-dry for 10 min (or dry using a hair dryer, set to cool air, for 1 min).
15. Store at RT till further use (within 1 week) (*see* **Note 8**).
    (a) A typical image using this type of protocol is shown in Fig. 1b, d.
16. Proceed with Subheading 3.5.

3.4 Histochemical Staining for Isolation of Inclusion Bodies (Protocol C)

1. Briefly wet the sections with sterile ultra-pure H_2O by pipetting it on the sections.
2. Incubate in eosin solution for 30 s.
3. Wash the slides 30 s in H_2O.
4. Wash the slides in ethanol 100% three times 30 s.
5. Air-dry for 10 min (or dry using a hair dryer, set to cool air, for 1 min).
6. Store at room temperature (RT) till further use (within 1 week) (*see* **Note 8**).
   (a) Figure 1c displays a typical staining obtained using this method.
7. Proceed with Subheading 3.5.

3.5 Laser Dissection Procedure

1. Place the PEN-foil slide holding the tissue section with the tissue facing down.
2. Depending on the availability and amount of material that has to be dissected, either load the cap with 30 μL of 1× reducing SDS loading buffer (diluted with M-PER lysis buffer), or place an adhesive cap in the cap holder below the specimen (*see* **Note 10**).
3. Proceed with Subheading 3.6 for protocols A and B or Subheading 3.7 for protocols C and D.

3.6 Laser Dissection Procedure (Protocols A and B)

1. To obtain approximately 40 μg grams of protein, a volume of 1×10^9 μm^3 has to be isolated. This is best to be divided over two separate eppendorf tubes (60 μL SDS loading buffer in total).
2. Select area(s) of interest and dissect the tissue using the LDM system as indicated by the manufacturer.
3. After tissue isolation freeze at −80 °C until further use.
4. Proceed to Subheading 3.8.

3.7 Laser Dissection Procedure (Protocols C and D)

1. Start with making a small hole in the PEN membrane at a location without tissue to let the air out (*see* **Note 11**).
2. Select shapes and dissect shapes as indicated by the LCM manufacturer.
3. When the isolated material is in reducing SDS sample buffer, store in −80 °C until further use.
4. When the isolated material is in an adhesive cap, then proceed with taking it up in reducing SDS sample buffer as follows:
   (a) Add 25 μL of reducing SDS sample buffer.
   (b) Incubate for 10 min at RT.
   (c) Pipet "up and down" repeatedly (5–10 times).
   (d) Transfer to a new Eppendorf tube.

(e) Check the adhesive cap under a microscope to convince yourself that all material is removed from the adhesive cap.

5. Store at −80 °C until further use.
6. Proceed to Subheading 3.9.

3.8 SDS-PAGE for Large Areas of Interest (Protocols A and B)

1. Let the sample defrost and warm up to RT.
2. Heat the samples to 95 °C and incubate for 5 min.
3. Let the samples cool down to RT.
4. -optional- incubate with 50 mM iodoactamide for 30 min at RT in the dark.
5. Separate protein based on their size on a NuPAGE® 4–12% Bis-Tris acrylamide gel (Invitrogen) using MOPS SDS running buffer (Invitrogen) according to the manufacturer's protocol.
6. After electrophoresis, remove the gel from the cassette.
7. Incubate the gel in gel fixing solution for at least 2 h.
8. Wash the gel in ultra pure sterile water for 10 min.
9. Incubate in the Coomassie blue solution for 10 min.
10. Wash the gel in ultra pure sterile H_2O for several hours, refreshing the H_2O repeatedly, until the gel is completely destained.
11. The gel can now be cut into the required number of fractions, which depends on the type of mass spectrometer used. For the LTQ-Orbitrap mass spectrometer we used 12 fractions. For Triple TOF 5600 system we used 2–4 fractions. For a Fusion Lumos Orbitrap mass spectrometer it is possible to quantify 4000 proteins from a single fraction using a 2–3 h HPLC gradient (*see* Subheading 3.3, **step 2** for SDS-PAGE of proteins in a single fraction).
12. Proceed with in gel trypsin digestion protocol (Subheading 3.10).

3.9 SDS-PAGE for Individually Isolated Cells or Inclusion Bodies (Protocols C and D)

1. Make a 10% gel, of 1 mm thickness with 10 wells as follows (for 10 mL):
   (a) Mix 3.3 mL of the 30% acrylamide, 1.5 mL 1.5 M Tris–HCl (pH 8.8) and 4.96 mL ultra pure H_2O.
   (b) Add APS 0.1 mL and TEMED 0.006 mL, mix gently, and directly pipette this into the gel cast as polymerization starts directly. Place the 10 well comb and wait for the gel to polymerize (typically <30 min).
2. Place the prepared gel in the Bio-rad electrophoresis system and fill up with TGS running buffer 1×.
3. Remove the comb.
4. Load the samples into the gel wells.

5. Let the proteins run into the gel by electrophoresis (150 V) until the sample is in the gel for a length of approximately 8–10 mm.
6. Remove the gel from the cask.
7. Incubate the gel in gel fixing solution for at least 2 h.
8. Wash the gel in ultra pure sterile water for 10 min.
9. Incubate in Coomassie blue solution for 10 min or longer if desired (*see* **Note 12**).
10. Wash the gel in ultra pure sterile H_2O for several hours, refreshing the H_2O repeatedly, until the gel is completely destained.
11. Cut out the gel piece containing the sample.
    (a) The gel piece can be divided into two pieces (high and low molecular weight) if desired.
12. Proceed with in gel trypsin digestion protocol Subheading 3.10.

3.10 In Gel Trypsin Digestion

1. Cut the gel piece into small fragments of approximately 1 mm^3 using a razor blade. We often use two razor blades, one to maintain the gel piece in place and the other to cut the gel. Transfer the gel fragments into a 1.5 mL Eppendorf tube.
2. Add 1 mL of 50 mM ammonium bicarbonate/50% acetonitrile to the Eppendorf tube, invert the tube, and put it on Table. This ensures a larger contact surface between the gel fragments and the destaining solution. Make sure that the gel fragments are at the bottom of the tube. Incubate for 2–3 h.
3. Remove the solution and discard. Capillary gel loading pipet tips can be used for this.
4. Add 0.5 mL 100% acetonitrile. The gel fragments should turn white and shrink within a few minutes.
5. Remove acetonitrile and discard.
6. Add 0.5 mL 50 mM ammonium bicarbonate, incubate for 5 min, and then add 0.5 mL 100% acetonitrile.
7. Invert the Eppendorf tube, place on Table, and incubate for 1 h or until the gel fragments are completely destained.
8. Remove the solution and discard.
9. Add 0.5 mL 100% acetonitrile and incubate for 5 min.
10. Remove acetonitrile and discard.
11. Dry the gel fragments in Speedvac for 5 min.
12. Add 3 mL 50 mM ammonium bicarbonate to one vial of Promega Trypsin/Lys-C Mix, and vortex.
13. Add 0.1 mL Trypsin/Lys-C Mix to each Eppendorf tube containing the gel fragments, wait 5 min to determine if more

trypsin/Lys-C Mix solution should be added to completely re-swell the gel fragments.

14. Add 0.1 mL 50 mM ammonium bicarbonate to the gel fragments, incubate overnight at 37 °C.
15. Transfer the solution from the Eppendorf tube containing the gel fragments into a new Eppendorf tube.
16. Add 0.2 mL 50 mM ammonium bicarbonate/50% acetonitrile to the gel fragments, vortex for 20 min.
17. Transfer this solution to the Eppendorf tube from **step 15**.
18. Dry the pooled solution in Speedvac. The samples can be stored in Eppendorf tube at −20 °C for weeks before mass spectrometry analysis.

3.11 Mass Spectrometry Analysis

1. HPLC and mass spectrometer from different vendors have their own specifications. The protocol described here is based on the use of an Ultimate 3000 LC system (Dionex) coupled to a Triple Tof 5600 mass spectrometer (Sciex). It serves merely as a guideline.
2. Redissolve the dried tryptic peptides in 7 μL of HPLC solvent A. Vortex for 15 min.
3. Transfer the solution to a sample vial, cap the vial, and place it in the autosampler tray.
4. Load 6 μL to the 5 mm Pepmap 100 C18 trap column (300 μm internal diameter, 5 μm particle size, Dionex).
5. Initiate the separation in the 100 μm (internal diameter) nano-LC column packed with C18 material (3 μm Altima C18 particle). The gradient is increased from 5% to 18% A in 88 min, to 25% at 98 min, 40% at 108 min, and to 90% at 110 min, at a flow rate of 400 nL/min.
6. Peptides are sprayed into the mass spectrometer, using an ion spray voltage of 2.5 kV, curtain gas at 35 p.s.i., nebulizer gas at 15 p.s.i., and an interface heater temperature of 150 °C. The MS survey scan range is m/z 350–1250 acquired for 250 ms. Select top 20 precursor ions for 85 ms per MS/MS acquisition, with a threshold of 90 counts. Dynamic exclusion was 16 s. Rolling CID function was activated, with an energy spread of 15 eV.
7. Process raw data with MaxQuant. The match-between-run option can be activated to minimize the number of missing values across the samples.

4 Notes

1. It is highly important that tissue is rapidly snap frozen in liquid nitrogen. When the freezing procedure is too slow, freezing artifacts will occur destroying the morphology and making the tissue crumble when cutting the tissue, especially at larger thickness.
2. Adhesive caps can also be used on a Leica system. These can be of practical use, especially when isolating individual cells of a type that is low abundant and therefore requires multiple days of laser dissection. When using adhesive caps no buffer is required to collect the sample in.
3. Check if the foil is intact. When the foil is damaged then fluid will get between the foil and the glass, rendering the slide unusable for LMD. This is especially relevant when isolating larger areas of tissue (protocols A and B).
4. For the analysis of larger areas of interest, usually thicker sections are better as that saves time in performing laser dissection and reduces the amount of (expensive) PEN-foil slides needed. Modern laser dissection microscopes, like the Leica LMD 6500, have sufficient laser power to cut through sections up to 50 μm. (Cutting sections on a cryostat does become increasingly difficult with increasing thickness. Increasing the temperature in the cryostat might help, but often one will have to settle with sections of a thickness below 50 μm.)
5. For the isolation of separate cells/inclusion bodies or other very small structures, sections are preferably thin, 10 μm or less. Thickness depends largely on the size of the objects that you wish to isolate, considering that when the section is thicker than the object of interest then tissue/material surrounding the object will be isolated as well, reducing the purity of your sample.
6. Typically we fix tissue in 100% ethanol, however, we experienced that some IHC stainings were incompatible with ethanol fixation, but were successful when fixing using 100% acetone. No negative effects were observed in the downstream LC-MS/MS analysis.
7. PEN-foil slides are easily damaged, and if so during the staining they can become unusable (also *see* **Note 3**). Therefore, treat the slides with great care.
8. Proteins in the tissue are, when dry, very resistant to degradation. It is often preferred to store the slides at RT instead of freezing them. Freezing introduces the risk of deposition of moisture, and freeze thaw cycles are best avoided. However, fast (within a week) processing of the sections is

recommendable. I.e. finishing the laser dissection and storing the samples immediately in reducing SDS buffer at −80 °C.

9. Usually, the antibody concentration used for a fast IHC staining is about tenfold higher as used in a normal overnight incubation for IHC. We recommend performing a test series prior to the actual experiment. Antibodies that produce a highly contrasted and specific staining will give the best chance of success, as the fast IHC protocol will produce a staining of lower quality than an overnight incubation. This combined with the absence of mounting medium will reduce the visual quality of a given specimen considerably. No blocking proteins are used as these are potentially picked up by the mass spectrometer, obscuring the results.
10. To decide for either the cap with SDS sample buffer or an adhesive cap is particularly valid when isolating separate cells/inclusion bodies (protocol C or D). For our experiments we found a number of 3000 cells/inclusion bodies to provide satisfactory results. Although even higher numbers will obviously result in more proteins that can be quantified as well as more reliable quantification. When a particular cell or structure of interest is very rare in the tissue, it can take several days to isolate sufficient cells for successful MS analysis. Our experience is that it is then useful to capture the material in adhesive caps. It is then quite well fixed in place and you can store it at RT and easily continue then next day with dissection using the same cap.
11. Usually, dissection of small structures such as separate cells, inclusion bodies, or protein aggregates is done at high magnification, for example using the 20× or the 40× objective. The PEN-membrane glass slide usually has some air between the glass and the foil. When selecting a number of shapes to be dissected you will find that when the first hole is made in the foil, the air will come out and the foil will move to the glass. Consequentially, your tissue section will be out of focus for all the remaining shapes, which will compromise the precision and effectivity of the dissection. This step is not necessary when metal frame slides are used.
12. Since only very little amount of protein is isolated the Coomassie staining will be very weak. Longer incubation time can increase the signal. However, complete lack of staining does not necessarily indicate absence of proteins. Successful quantification of a high number of proteins can still be realized using a very sensitive mass spectrometer.

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Chapter 22

Laser-Capture Microdissection and RNA Extraction from Perfusion-Fixed Cartilage and Bone Tissue from Mice Implanted with Human iPSC-Derived MSCs in a Calvarial Defect Model

Xiaonan Xin, Xi Jiang, Alexander Lichtler, Mark Kronenberg, David Rowe, and Joel S. Pachter

Abstract

Laser-capture microdissection (LCM) coupled to downstream RNA analysis poses unique difficulties for the evaluation of mineralized tissues. A rapid protocol was thus developed to enable sufficient integrity of bone and cartilage tissue for reliable sectioning, while minimizing RNA loss associated with prolonged decalcification and purification steps. Specifically, the protocol involves pump-assisted, cardiac perfusion-fixation with paraformaldehyde, and moderate digestion of LCM-acquired tissue with proteinase K followed by DNase treatment and separation of RNA using magnetic beads. Reverse transcription and cDNA synthesis are performed immediately after RNA purification, without need for further protein removal.

Key words Calvarial defect model, Cartilage, Mesenchymal stem cell, Pluripotent stem cell

1 Introduction

Laser-capture microdissection (LCM) allows for selective acquisition of distinct subpopulations of cells from a heterogeneous background [1–3]. While LCM can be used to retrieve designated whole cell targets from ex vivo culture preparations, it has found its widest application to the removal of cell "profiles"; i.e., slices of cells, from their in situ environments within tissue sections. And though most tissues are readily amenable to LCM—e.g., use of fresh-frozen samples and routine glass slides—mineralized ones such as bone present particular challenges due in large part to difficulties in sectioning and adherence to slides. This has largely

Xiaonan Xin and Xi Jiang contributed equally to this work.

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restricted the use of LCM to the analysis of bony tissue at early developmental stages, when mineralization is limited [4].

To overcome these obstacles, adult bone is often decalcified prior to sectioning, but this too is not without consequences and caveats. Decalcification protocols are often long in duration, e.g., 3–15 days, which in turn significantly increases risk to RNA integrity [5]. Cross-linking fixatives have been introduced to mitigate RNA loss as well as to improve morphology, but can themselves compromise RNA quality [6, 7]. Immersion fixation, in particular, further suffers from not being able to deliver fixative to all regions of tissue at the same rate [8]. For example, optimal preservation by formaldehyde solutions requires an excess of 20 times the volume of tissue [6]. And digestion conditions to reverse cross-linking can fracture RNA and pose impediments to delicate enzymatic reactions employed in downstream platforms [9]. Lengthy RNA purification procedures to eliminate contaminants can further depress nucleic acid yield and be cumbersome when dealing with numerous, small volume samples.

A favorable LCM protocol for bone is thus one that will enable efficient sectioning in the absence of prolonged decalcification, while maximizing RNA integrity. Accordingly, we have developed a facilitated protocol for LCM of adult bone tissue using pump-assisted, cardiac perfusion-fixation with 3.2% buffered paraformaldehyde, coupled to post-LCM in situ tissue digestion, followed by magnetic bead separation of RNA and reverse transcription.

This LCM protocol has most recently been applied to a novel, bone model system established by this group [10]. Specifically, the model system involves the following steps: (1) deriving-induced pluripotent stem cells (iPSC) from patients, control individuals, and potentially from individuals with identified genetic traits that are associated with a skeletal phenotype such as high or low bone mass; (2) differentiating these cells into a mesenchymal stem cell-like (MSC) population that has the potential to produce cartilage and bone, and (3) implanting these cells into a mouse calvarial defect model. In this model system, our MSCs produce proliferating stage cartilage, hypertrophic and mineralized cartilage, and bone. Although significant amounts of these cells types are reproducibly produced, the structures of the skeletal elements generated are not consistent enough to dissect out specific tissues on a macro scale for analysis and, thus, LCM affords unique opportunities for analysis. Here, we present our most recent LCM protocol for adult bony tissue that could potentially yield RNA of sufficient quantity and quality for amplification and deep sequencing using Illumina technology.

2 Materials

2.1 MSCs

Human iPSCs were differentiated into MSCs and implanted into 3.5 mm diameter calvarial defect in a HEALOS scaffold (*Xin et al., manuscript in preparation*). If implants are allowed to develop for 6 weeks before sacrifice, the implants contain large amounts of human cartilage. If the implants are allowed to develop longer, the cartilage progressively becomes hypertrophic, mineralizes and is replaced by bone.

2.2 Perfusion-Fixation

1. Stock paraformaldehyde 32% solution, EM grade (methanol-free), Electron Microscopy Sciences.
2. 10× Phosphate-buffered saline (PBS), pH 7.4, Bio-Rad.
3. 1 volume 10× PBS: 8 volumes double distilled water.
4. Masterflex L/S peristaltic pump with 96410-13 tubing speed setting 5.

2.3 Tissue Sectioning

1. Cryostat, CM 3050, Leica.
2. Shandon Cryomatrix, Thermo Scientific, or O.C.T. Compound, Tissue-Tek, Sakura.
3. Disposable base molds, Fisherbrand, 24 × 24 × 5 mm, Thermo Fisher.
4. CryoJane ECU Tape Transfer System, Leica, which is installed in the Cryostat.
5. Cryojane Cryostat Frozen Sectioning Aid Adhesive Coated Slides (CFSA) CS 4×, Leica.
6. CryoJane AdhesiveTapeWindows, Leica.

2.4 LCM

1. ArcturusXT LCM System, with or without epifluorescence optics, Thermo Fisher.
2. CapSure® Macro LCM Caps (48 caps), Life Technologies.

2.5 RNA Extraction

1. Oven set to 55 °C (an Autoblot® microhybrization oven [Bellco Glass, Inc.] is used by this group). Inside the oven is a block wrapped in plastic tape, sticky side up, onto which a microfuge tube, containing a Capsure® Macro LCM Cap, can be placed so that LCM-acquired tissue can be fully exposed to Modified Lysis Buffer for efficient RNA extraction (Fig. 1).
2. TempBlock or water bath set to 75 °C.
3. Direct cDNA Cell Lysis buffer, Signosis.
4. Proteinase K, AmericanBio.
5. Modified Lysis Buffer (MLB).

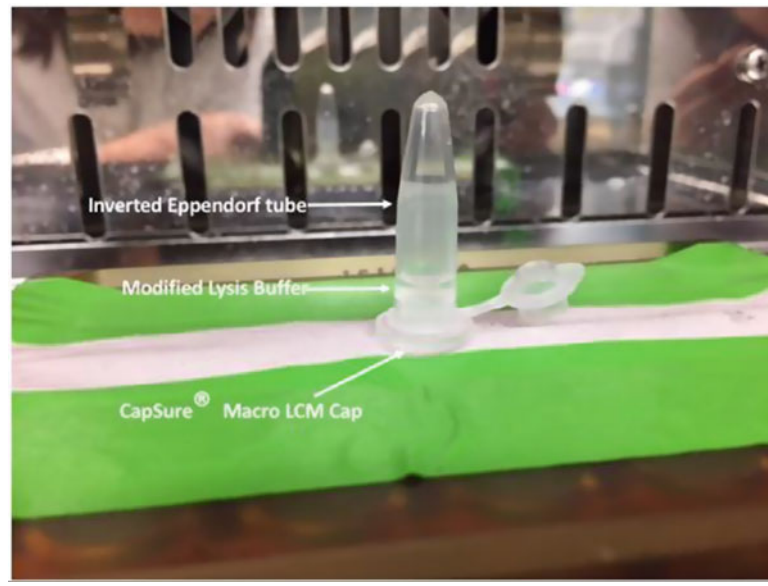

Fig. 1 Extraction of LCM-acquired material. Once sufficient material is acquired by LCM, Modified Lysis Buffer, preheated to 55 °C, is added to a sterile Eppendorf tube. The LCM cap, containing the tissue, is snapped into the top of the Eppendorf tube. The tube is then inverted and placed in the pre-warmed oven at 55 °C for 30 min

Preparation of Proteinase K stock solution: Dissolve Proteinase K in 10 mM Tris-HCl, pH 7.5, 20 mM $CaCl_2$, 50% Glycerol at 20 mg/ml. Proteinase K from AmericanBio comes with 100 mg powder per bottle, so add 5 ml buffer to the bottle, mix to dissolve, and store at −20 °C. Proteinase K from this supplier has 30 units of activity/mg; to use proteinase K from other suppliers it may be necessary to adjust the mg/ml to achieve 600 units/ml. Preparation of MLB: Thaw 40 μl Signosis Cell Lysis buffer per cap (plus enough for 1 additional cap) immediately before use, and keep on ice. Add 1/50 volume proteinase K, for a final concentration of 0.4 mg/ml.

6. RNaseAway, Life Technologies.
7. TURBO DNA-free DNase treatment kit, Life Technologies.
8. RNAClean XP magnetic beads, Beckman Coulter.
9. 100% ethanol, highly purified (any reputable supplier).
10. RNase-free water (any reputable supplier).
11. Magnet unit for use with 0.2, 0.5, or 1.5 ml microfuge tube. We use a MiniMacs apparatus, Miltenyi Biotec, though the Agencourt SPRIPlate 96R Ring Magnet Plate, Beckman Coulter, may have some advantages.

2.6 cDNA Synthesis

1. dNTPs, any reputable supplier, such as Roche, Promega, NEB, Life Technologies.
2. Superscript III Reverse Transcriptase, Life Technologies.
3. Random hexamers, any reputable supplier.
4. NuGEN Ovation RNA-Seq FFPE System.

3 Methods

3.1 Preparation of Tissue/Slides for LCM

1. Mice are cardiac perfused with ~10 ml of PBS, pH 7.4, followed by 50 ml of 3.2% PFA in PBS, using a Masterflex L/S peristaltic pump at speed setting 5.
2. Calvaria containing implants are dissected out and fixed for 2–4 h in 3.2% PFA in PBS at 4 °C.
3. The calvaria are removed from the PFA, briefly dried, and embedded in Shandon Cryomatrix in a base mold, snap frozen for at least 20 min in methyl-butane that had been prechilled on dry ice, and stored at −80 °C until use. The samples should be oriented in the mold so the sections will be cut in the desired plane of the specimen. In our case the calvaria with the human cell implant is placed to give coronal sections through both the mouse calvaria and the human implant.
4. Before sectioning, the tissue blocks are removed from the mold and frozen onto the specimen disc in Cryomatrix and placed on the specimen head, all in the Cryostat chamber.
5. Thick (40–60 μm) sections are cut until the appropriate level of the specimen is reached. Check using a microscope to make sure the correct level is reached.
6. The cryostat is adjusted to give thinner sections; we usually use 7 μm sections.
7. A precut piece of CryoJane Adhesive Tape is chilled in the Cryostat chamber, placed on the specimen, a chilled roller is rolled over the tape to make sure it has good contact with the sample.
8. The section is cut, the tape is placed with the section down on a CryoJane slide that had been placed on the CryoJane slide station within the Cryostat chamber, the roller is rolled over the tape to ensure good contact. A gloved finger is used to press briefly onto the tape to warm it briefly (a few sec). Alternatively, the roller is not used, and only the gloved finger is used to gently press down on the tape. If more than one section is put on one slide they are all done in succession.

9. The slide is put in the CryoJane UV station to fix the section onto the slide. The on switch is activated twice, with 20 s between activations.
10. The tape and section are again briefly warmed with a gloved finger, and the tape is gently peeled off, leaving the tissue section on the slide. The brief warming is necessary to prevent peeling the section off with the tape, and it is probably necessary to experiment to determine the minimum time to warm the slide before peeling the tape off and still get good transfer to the slide.
11. The slide is then dipped in a graded series of ethanol in RNase-free water, 75% for 30 s, 95% for 30 s, 100% 2× for 1 min, and 100% xylene 2× for 1 min each, store in xylene until right before LCM.

3.2 LCM

1. Immediately before LCM, dry the slide in the chemical hood until the xylene has evaporated.
2. Put the slide on the LCM microscope stage. It is important to carry out the LCM procedure as quickly as possible after letting the xylene evaporate, to avoid letting the section rehydrate by absorbing water from the air. It is also valuable to run a dehumidifier in the room containing the LCM instrument and isolate that room as much as possible, to keep the air as dry as possible.
3. Identify the regions containing specific tissue types to be captured. In our case, since our sections are not decalcified, we identify mineralized tissues as dark areas, because the mineral blocks the light. Mineralized cartilage has a distinct appearance because the large hypertrophic chondrocyte cell bodies form distinctive large round holes in the mineral layer, while bone is generally more densely mineralized, and does not have the round holes (Fig. 2).
4. Use the computer mouse to outline the area containing the tissue to be captured. Then activate the UV laser to cut around the outline, and observe whether the tissue is completely cut, which can usually be detected as a visible line through the matrix. Mineralized tissue is particularly resistant to the UV laser. If cutting is not complete, it may be helpful to repeat the UV laser cutting multiple times. It is important to not try to outline an area that is too small, because the UV laser can damage RNA that is too close to the laser cut line.
5. When satisfactory cutting is achieved, activate the IR laser to lift the target tissue and attach it to the LCM cap. You should adjust the size of the IR spot depending on the size and shape of the area you want to capture, to avoid producing IR spots that cross the UV cut line.

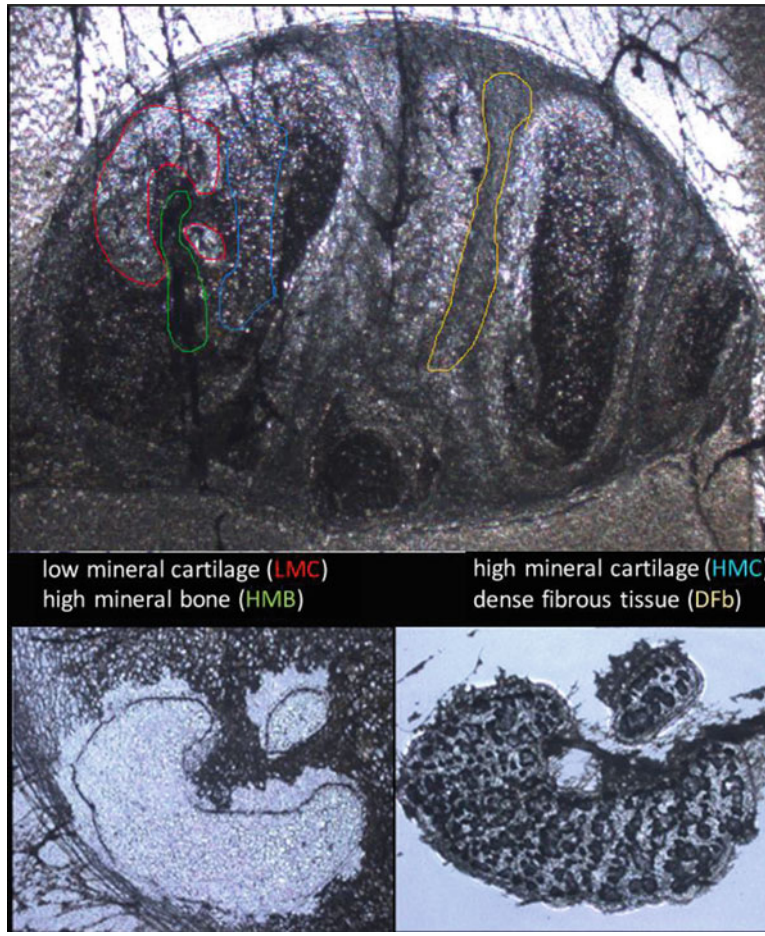

Fig. 2. LCM retrieval of specific cell types from human iPSC-derived skeletal tissues produced in our mouse calvarial defect model. The upper panel shows the outlines drawn with the mouse around four different tissue types. The identities of the tissues in this image were determined based primarily on the mineral density observed under transmitted light optics, but we have also used multiple staining modalities, including H&E, toluidine blue, safranin O, alkaline phosphatase, human-specific bone sialoprotein antibody, and darkfield imaging on similar sections to identify these tissue types (data not shown). Low mineral cartilage (LMC) refers to unmineralized tissue containing cells with chondrocyte morphology. High mineral cartilage (HMC) refers to mineralized areas with large round cell with hypertrophic cartilage morphologies. High mineral bone (HMB) refers to more densely mineralized tissue with cells that lack the distinctive hypertrophic chondrocyte morphology, and dense fibrous tissue (DFb) refers to areas with a fibrous appearance that are distinct from looser fibrous areas. The bottom panels show, as an example, the LMC sample on the cap (lower right panel), and the remaining section (lower left panel). Note that there is some tissue removed outside of the UV laser cut even after multiple passages of the laser, because of the toughness of the matrix

6. Activate the cap removal function of the LCM instrument, then follow the procedures to inspect the material removed on the cap and what is left behind, to make sure the appropriate area is taken.

7. If it is desired to obtain more of the same type of tissue, and there is more of the desired tissue type on the slide as well as room on the cap, you can reposition the cap and repeat the process.
8. Once enough sample has been obtained, remove the LCM cap from the instrument, and either place the cap in an appropriate sized RNase-free microfuge tube, freeze it on dry ice and store at −80 °C for later use, or proceed to the RNA extraction protocol.

3.3 RNA Extraction (see Note 1)

1. Preheat oven with tape-covered block at 55 °C and TempBlock or water bath at 75 °C.
2. Prepare sufficient MLB for the number of caps you are going to process, plus one sample extra to allow for pipetting inaccuracy. Immediately before use, pre-warm the MLB at 55 °C.
3. Remove the LCM cap from the microfuge tube (if the tube is frozen, it may help to use a cap removal device, which is available from companies such as NEB), add 40 μl pre-warmed MLB to the tube, snap the LCM cap back on tightly, and use a quick snap of the wrist to bring the MLB to the top of the tube, so it is in contact with the LCM cap. Place the tube in the oven upside down on the tape, and incubate for 30 min.
4. Spin the tube briefly in a microfuge at maximum speed to bring the MLB to the bottom of the tube, then incubate at 75 °C for 20 min to inactivate the proteinase K. Proceed to DNase step, or quick freeze and store at −80 °C until further processing.

3.4 DNase Treatment (see Note 2)

1. Thaw the 10× DNase buffer and DNase Inactivation Reagent from the TURBO DNA-free kit. Transfer 15 μl of extracted RNA to a fresh tube.
2. Add 1.7 μl 10× DNase buffer and 0.5 μl DNase enzyme to the tube and mix. If processing multiple samples, make a master mix containing these volumes of buffer and enzyme times sample number +1, mix, and add 2.2 μl of the mix to each sample.
3. Incubate at 37 °C for 30 min.
4. Add 2 μl of DNase Inactivation Reagent, which contains beads that bind DNase. Mix by flicking carefully, and incubate at room temperature for 2 min (periodically mixing to keep the beads suspended).
5. Spin at 10,000 × *g* for 1.5 min. Transfer the supernatant to a fresh tube (you should be able to remove 11–15 μl without disturbing the beads). Proceed directly to Magnetic Bead Purification or freeze.

3.5 Magnetic Bead Purification

1. Warm RNAClean XP magnetic beads to room temperature; this is necessary to ensure maximum efficiency of binding of RNA to the beads. Shake bottle of beads gently to resuspend them.
2. Add 1.6–1.8 volumes of the beads to the RNA sample. For 15 μl of RNA, this is 27 μl, but this procedure can be scaled up substantially to purify RNA from larger volumes, as long as the proportion of beads to RNA is kept the same. The microfuge tube size can be adjusted to accommodate larger volumes, as long as the magnetic apparatus you have is compatible. After the beads are added, flick the tube to mix the beads with the RNA and let it sit for ~10 min to allow the RNA to bind the beads, with occasional flick-mixing to keep the beads suspended.
3. Place the tube in the magnet, and wait ~5 min, or until the beads have moved to the side of the tube next to the magnet.
4. Remove most of the supernatant. It is best to leave a few microliters in the tube to avoid removing some of the beads, which would decrease RNA yield.
5. Wash 3× with 200 μl of 70% ethanol. It is important to make the 70% ethanol immediately before using, making sure to derive from a recently opened or tightly closed bottle of ultra-pure 100% ethanol. Long exposure of ethanol to air allows uptake of water from the air, and ethanol concentrations lower than 70% may lead to partial elution of RNA from the magnetic beads. The washes are done by gently adding the ethanol to the tube, ensuring that the beads have not been washed away from the magnet. The ethanol is then removed with the tube still on the magnet, putting the pipette down to the bottom of the tube, avoiding the magnetic beads. After the third wash, the last few microliters should be carefully removed to speed up drying. If a large volume of RNA is being purified in a large microfuge tube, and depending on the magnetic apparatus being used, it may be necessary to increase the volume of 70% ethanol to cover all of the magnetic beads.
6. Allow the ethanol to dry thoroughly for up to 20–30 min.
7. Elute by taking the tube off the magnet and adding water or dilute buffer such as TE (10 mM Tris-HCl pH 7.5, 1, or 0.1 mM EDTA). Resuspend the beads by pipetting up and down or by gently flicking the tube. Let the RNA elute for ~3 min, putting the tube back on the magnet for ~2 min or until all of the beads are drawn to the magnet. Remove the supernatant. The elution volume can be adjusted depending on the downstream application for the RNA. If maximum yield is of priority, you can elute in a larger volume such as 30–40 μl. Multiple elutions can be done to increase yield. For maximum

concentration, elution in a smaller volume can be done, with some decrease in yield. Elution in as little as 10 μl can yield sufficient of RNA for some applications.

4 Notes

1. This protocol obviates the need for protracted RNA purification steps from fixed tissue acquired by LCM.
2. DNase treatment of extracted tissue effectively eliminates detectable genomic DNA contamination (Fig. 3).
3. RNA from different tissues are enriched in mRNA that is specifically expressed in those tissues (Fig. 4).
4. RNA amount may be estimated from LCM-acquired tissue, as shown using quantitative Real-Time PCR with 18 ribosomal and rPL19 primers and standard curves (Fig. 5).

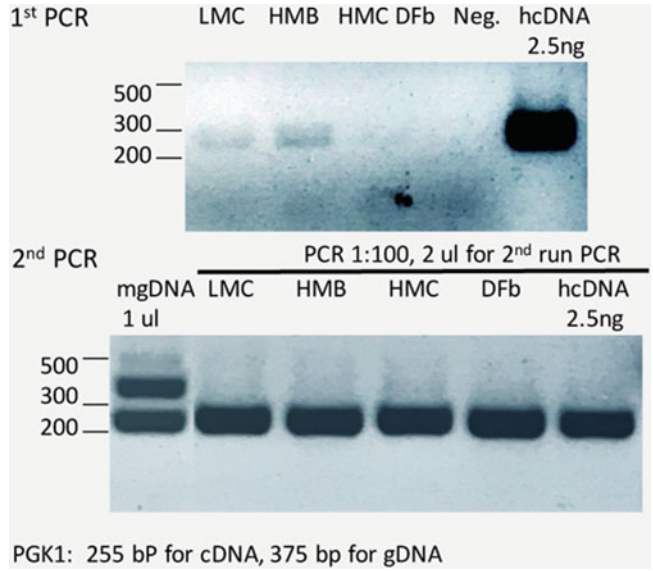

Fig. 3 Confirmation of DNAse elimination of DNA contamination of samples. cDNA was generated from RNA purified using our procedures. PGK1 PCR primers produce a 255 bp band from cDNA or processed pseudogenes, and a 375 bp band from the functional gene with and intron. The top panel shows the first round of RT PCR of RNA from the same tissues shown in Fig. 2; in some cases, the bands are very faint. To improve sensitivity, a second round of PCR was done, which produced a robust band of the size produced by the cDNA, but no band was produced of the size produced by the intron-containing genomic DNA

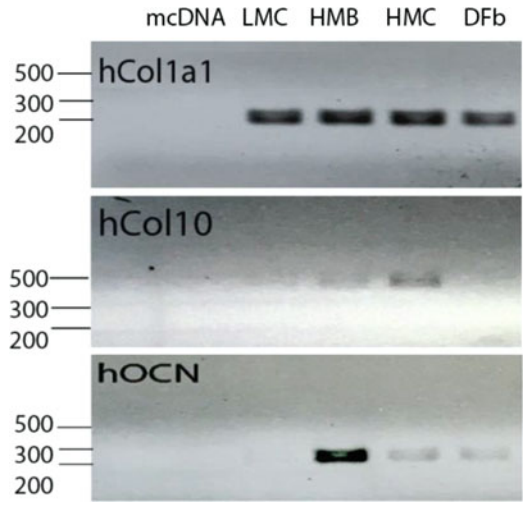

Fig. 4. PCR analysis of tissue-specific RNAs from material obtained by LCM from calvarial defect model. Following extraction of calvarial tissues acquired by LCM (Fig. 2), cDNA was generated from RNA purified using our procedures using SuperScript III (Life Technologies), following the manufacturer's instructions. We designed human specific primers for COL1A1, COL10 and osteocalcin (OCN) genes. As expected, all tissues express the COL1A1 gene, which was confirmed using COL1a1 antibody staining (data not shown). COL1A1 is expressed in chondrocytes because the cartilage made in our model is fibrocartilage, which is known to express the COL1A1 gene. COL10 collagen is a marker for hypertrophic chondrocytes, so it is expected that it would be most highly expressed in HMC tissue. Osteocalcin is a marker for osteoblasts, so it is expected that it would be most highly expressed in HMB tissue (*see* **Note 3**)

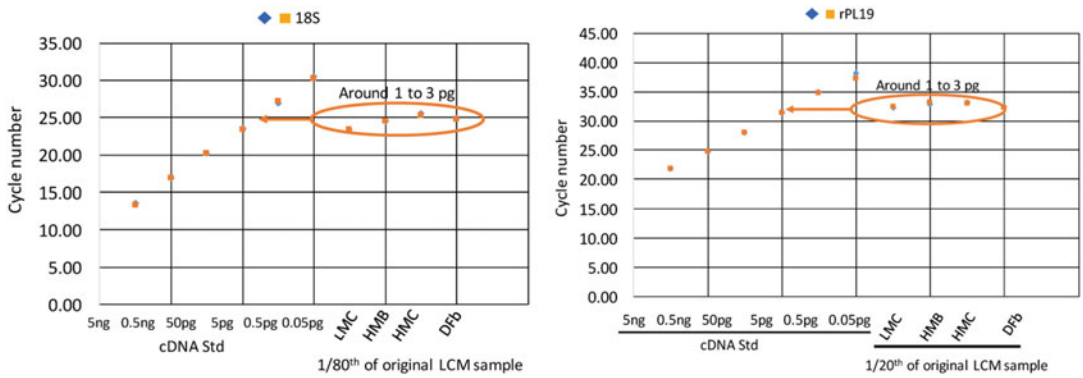

Fig. 5. Quantitative Real-Time PCR allows estimation of the amount of RNA extracted from LCM samples. BioRad SsoFast EvaGreen Supermix real-time PCR mastermix was used in a BioRad CFX96 machine. Standard curves for 18S ribosomal and rPL19 ribosomal protein were produced using cDNA produced from cultured human cells using Trizol. Comparing the CT values of the LCM samples to the standard curve, and multiplying by the dilution factor we used for the different primer sets, indicated that we obtained between 1 and 3 pg of RNA from our samples (*see* **Note 4**)

Acknowledgments

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Chapter 23

Laser Capture Microdissection-Based RNA-Seq of Barley Grain Tissues

Ronny Brandt, Martin Mascher, and Johannes Thiel

Abstract

Spatiotemporal patterning throughout the plant body depends to a large degree on cell- and tissue-specific expression of genes. Subsequently, for a better understanding of cell and tissue differentiation processes during plant development it is important to conduct transcript analyses in individual cells or tissue types rather than in bulk tissues. Laser capture microdissection (LCM) provides a useful method for isolating specific cell types from complex tissue structures for downstream applications. Contrasting to mammalian cells, the texture of plant cells is more critical due to hard, cellulose-rich cell walls, large vacuoles, and air spaces which complicates tissue preparation and extraction of macromolecules, like DNA and RNA. In particular, developing barley seeds (i.e. grains) depict cell types with differences in osmomolarity (meristematic, differentiating and degenerating tissues) and contain high amounts of the main storage product starch. In this study, we report about methods allowing tissue-specific transcriptome profiling by RNA-seq of developing barley grain tissues from low-input RNA amounts. Details on tissue preparation, laser capture microdissection, RNA isolation, and linear mRNA amplification to produce high-quality samples for Illumina sequencing are provided. Particular emphasis was placed on the influence of the mRNA amplification step on the transcriptome data and the fidelity of deduced expression levels obtained by the developed methods. Analysis of RNA-seq data confirmed sample processing as a highly reliable and reproducible procedure that was also used for transcriptome analyses of different tissue types from barley plants.

Key words Laser capture microdissection, Barley seeds, Cryosectioning, mRNA amplification, Global transcriptome analysis, RNA-seq

1 Introduction

Laser-assisted microdissection systems in combination with large-scale gene expression analysis had been developed and frequently used in mammalian systems [1, 2]. In plants, these applications were used to monitor gene expression profiles in a variety of different tissue types, like epidermal cells, vascular tissues, roots, shoot apical meristems, embryo cells, and seeds of different species [3–6]. In the last few years, LCM-based approaches coupled to next-generation sequencing (NGS) methods have been used for global transcriptome profiling of distinct root cell types, shoot

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apical meristems, phloem cells, reproductive meristems, cells of the female gametophyte, and seed tissues of different plant species [7–13]. Mostly all of the studies in plants use chemical fixation and paraplast embedding for tissue preparation prior to laser microdissection. Cryosections from frozen tissues provide the highest yield and the best quality of RNA extracted from microdissected tissues and is the standard method for animal and human tissues. In plant tissues, cryosectioning would also represent the method of choice, because any chemical pretreatment (fixation) has an influence on RNA integrity and decreases the amount of RNA that can be extracted. The main problem for using of cryosections is the reduced morphological resolution compared to fixed tissues which should be high enough to allow precise target isolation, especially when fine structures and small cell/tissue types have to be isolated. Due to enormous differences in the structure of plant tissues, no standard protocol for tissue preparation exists.

Grain development in barley is initiated by double fertilization which originates the diploid zygote and the triploid nucleus of the endosperm mother cell. Endosperm development starts with subsequent, numerous rounds of divisions of the triploid endosperm nucleus which results in the formation of a multinucleate syncytium surrounding the large endosperm vacuole. Cellularization of the syncytium starts in the ventral part of the grain at the intersection of maternal and filial tissues and generates the highly specialized endosperm transfer cells (ETCs). Around 3–4 days after flowering (DAF), cellularization of the ETC region is completed whereas in the remaining part of the syncytium cell wall formation is just initiated. As little is known about regulatory pathways involved in early endosperm differentiation, we wanted to get information about signals specifying ETC identity in comparison to the remaining syncytium. Comparative, genome-wide transcriptome analysis between the earliest endosperm compartments revealed activated differentiation and signaling pathways for ETC specification.

In this chapter, we describe suitable methods allowing tissue-specific RNA-seq from cryosectioned barley grains. Cryosections preserve a cellular resolution adequate for precise isolation of target tissues of barley grains—even at critical, early differentiation stages—and provide sufficient amounts of RNA for subsequent mRNA amplification. A further improvement compared to previously developed protocols for microarray-based transcriptome analysis [14] is that due to higher amounts of extracted RNA a second round of mRNA amplification can be avoided, which results in a decreased bias of the generated transcriptome data. One focal point during methodical development was to get information about the influence of mRNA amplification on gene expression levels compared to a non-amplified sample preparation starting with high amounts of total RNA. After the generation of high-

quality antisense RNA (aRNA) sample processing was incorporated into the standard TruSeq v2 protocol. Using the developed methods for RNA-seq, we were able to identify distinct groups of genes which might be involved in cell specification and the first steps of endosperm cellularization in barley.

2 Materials

1. Tissue-Tek® O.C.T.™ compound (Sakura Finetek Europe B. V., Zoeterwoude, Netherlands).
2. RNAse-free MMI Membrane Slides, 1.5 μm.
3. Cryostat (CryoStar™ NX70 Cryostat, Thermo Fisher Scientific, Walldorf, Germany).
4. mmi CellCut Plus® (MMI, Eching, Germany).
5. 0.5 mL MMI Isolation Caps.
6. RNase AWAY® (Roth, Karlsruhe, Germany).
7. RNA isolation reagents, Absolutely RNA Nanoprep Kit (Agilent).
8. Linear amplification kit, MessageAmpTM II aRNA Amplification Kit (Ambion).
9. Programmable thermo cycler and regulated hybridization oven.
10. Illumina ® TruSeq™ RNA Sample Preparation Kit v2 (Illumina, San Diego, USA).
11. Illumina HiSeq 2000 system.

3 Methods

In the following, methods are described for tissue preparation (1); laser capture microdissection (2); extraction of total RNA (3); T7-based amplification of mRNA and incorporation into TruSeq™ RNA Sample Preparation Kit for library production (4); and quality assessment of RNA-seq data (5).

3.1 Tissue Preparation

The protocol in this chapter describes the preparation of cryosections for laser capture microdissection (*see* **Note 1**; Fig. 1).

1. Barley grains at 3–4 DAF were frozen in liquid nitrogen and transferred to a cryostat cooled down to −20 °C. Using a razor blade, the middle part of the grain was cut out and glued onto the sample plate by O.C.T™ compound.

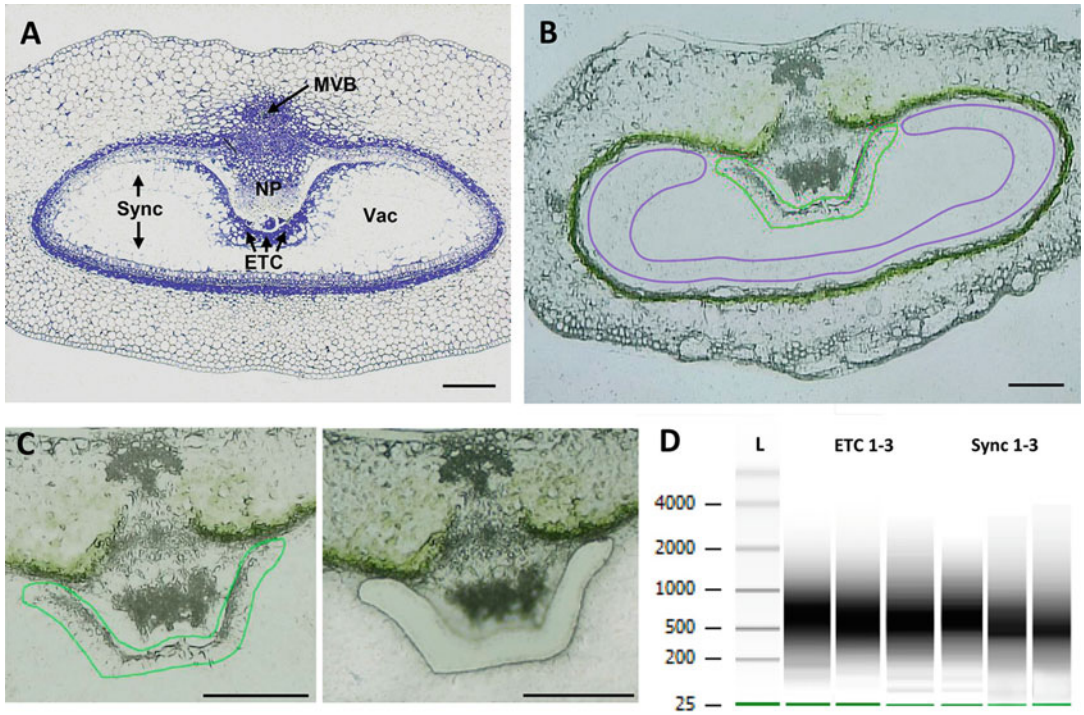

Fig. 1 Barley grain tissues at 3 days after flowering (DAF), laser-capture microdissection (LCM), and quality of antisense RNA (aRNA) used for RNA-seq. (**a**) Median cross-section of a barley grain just at the start of cellularization of the syncytial endosperm (3/4 DAF). Grains were fixed in PFA and embedded in BMM medium, arrows indicate selected grain tissues. *ETC* endosperm transfer cells, *MVB* main vascular bundle, *NP* nucellar projection, *Sync* syncytium at the start of cellularization, *Vac* endosperm vacuole. (**b**) Representative cryosection of a barley grain at 3 DAF. Despite that the cellular resolution cannot reach the quality of fixed sections (*see* **a**), all tissues are visible and can be targeted by LCM. Encircled in green—ETCs, in purple—Sync surrounding the endosperm vacuole. (**c**) Magnification of the ETC-region; marking of ETCs (left), section after cutting and isolation of ETCs by LCM (right). (**d**) Gel-like images of aRNA synthesized from low amounts of total RNA (up to 5 ng) of endosperm tissues as deduced by microcapillar electrophoresis. aRNA displayed a size distribution from 200 to 2000 nucleotides after the first round of amplification, with a maximum between 500 and 800 nucleotides. Size distribution indicates less RNA degradation and intact mRNA copies as it is known from shock-frozen tissues. Bars = 150 μm

2. Serial cross sections of 20 μm thickness were prepared using a cryostat and immediately mounted on PEN membrane slides in the cryostat chamber.
3. The PEN membrane slides were stored for 5–7 days in the cryostat at −20 °C until complete dryness.
4. Prior to microdissection, dry cryosections were adapted to room temperature in a glass desiccator with silica gel for 30 min (*see* **Note 2**).

3.2 Laser Capture Microdissection

1. The MMI Laser Cell Cut system was used to isolate specific tissues from cryosections of barley grains.

2. The laser beam (diameter <1.0 μm) was adjusted for cutting. Control software mmi CellTools® was used as a graphic tool for targeting of cells. Sections from the syncytium and endosperm transfer cells (Fig. 1c) were collected in the adhesive lid of 0.5 mL MMI Isolation Caps by lowering and lifting the cap.
3. Typically between 30 and 40 sections were processed per sample. One advantage of using PEN membrane slides is that tissues are cut out completely, so that efficiency of tissue-transfer to the lid of reaction tubes can easily be controlled by a microscope.

3.3 RNA Isolation

There are a number of commercially reagent systems available for isolating micro-amounts of RNA. Most of them are column-based systems avoiding organic extraction, phase separation, and precipitation steps, thus probably minimizing loss of RNA yield during these steps. The RNA purification described here is done with the Absolutely RNA nanoprep Kit using columns with a silica-based fiber matrix for RNA binding. The procedure has been conducted after the manufacturer's instructions with slight modifications which are described in detail.

1. Prepare lysis buffer by adding 0.7 μL β-mercaptoethanol to 100 μL lysis buffer for each sample. Lysis buffer is preheated to 60 °C in a heating block for 10 min.
2. Add 100 μL of lysis buffer directly on the isolated tissues in the cap and vortex the tubes upside-down for 2 min. Sections in lysis buffer are incubated for 15 min at 60 °C and vortexing is repeated for 2 min (*see* **Note 3**).
3. Add an equal volume of 70% ethanol (usually 100 μL) to the cell lysate and vortex thoroughly for some seconds. Transfer the mixture immediately to an RNA-binding nano-spin column seated in a 2 mL collection tube and spin for 1 min at ≥12,000 × *g*.
4. Discard the filtrate and place the column in the same tube. Add 300 μL of 1× low-salt wash buffer onto the column and spin the sample in a centrifuge for 1 min at ≥12,000 × *g*. Retain the tube, discard filtrate, and spin column again for 2 min (≥12,000 × *g*) to dry the filter matrix. It is important that the filter matrix is completely dry; otherwise, remaining ethanol residues would inhibit on-column DNase digestion. Place the column in a new 2.0 mL tube.
5. Add 24 μL of DNase solution (4 μL DNase I reconstituted in 20 μL DNase Digestion Buffer) directly on the fiber matrix of the column. Incubate at 37 °C for 15 min.
6. Add 300 μL of 1× high-salt wash buffer and spin in a centrifuge for 1 min at ≥12,000 × *g*. Retain spin cap and discard

filtrate. Perform two additional washing steps with 300 μL of 1× low-salt wash buffer as described above.

7. Discard filtrate and transfer column to a dry 2.0 mL tube. Spin column again for 3 min (≥12,000 × *g*) to dry the fiber matrix.
8. Transfer the column to a new 2.0 mL collection tube and add 12 μL of preheated elution buffer directly on the fiber matrix. The elution buffer has to be warmed to 60 °C for 10 min to increase the yield of RNA. The sample is incubated for 5 min at room temperature.
9. Spin the sample in centrifuge for 5 min at ≥12,000 × *g*. The purified RNA (~11 μL) is in the eluate in the collection tube. One microliter can be used for the estimation of RNA amount and/or quality, the remaining ~10 μL are used for subsequent amplification steps.

3.4 Linear Amplification of mRNA and Preparation of TruSeq Libraries

Up to 5 ng of total RNA could be extracted from 30 to 40 microdissected specimens (*see* **Note 4**). One round of in vitro transcription is sufficient to generate minimum amounts of aRNA (at least 100 ng) for RNA-Seq. In our protocol, we used the MessageAmpTM II aRNA Amplification Kit from Ambion based on the RNA amplification protocol developed from Eberwine and Van Gelder [15]. Using this reagent system, each sample yielded more than 200 ng of aRNA after the first round of amplification. Amplified aRNA displayed a size distribution between 200 and 2000 nucleotides with a maximum at 500–800 nucleotides implying less degradation during the sample preparation procedure (Fig. 1d). The procedure has been conducted after the manufacturer's instructions, but throughout the protocol some points should be remembered: Consistency in workflow is very important for amplification experiments. Standardize reaction incubation times and prepare master mixes for every step of the amplification procedure to minimize effects of pipetting errors. We recommend using a calibrated hybridization oven for prolonged 37 or 42 °C incubations and a calibrated thermal cycler for the 16 °C second strand synthesis reaction incubation. Because this step is very sensitive to temperature and temperatures above 16 °C compromise the yield of double-stranded cDNA, it is very important to use either a thermal cycler with a regulated lid temperature that matches the block temperature or not to close the lid if a static system is used. In general, condensation in the reaction tubes during any of the incubations leading to changes in the composition of reaction mixtures should be strictly avoided.

3.5 Reverse Transcription to Synthesize First Strand cDNA

1. Add 1 μL T7 oligo dT primer to the eluted RNA (11 μL) in a fresh 0.5 mL tube. Mix the reagents, incubate at 70 °C for 10 min, spin briefly and place on ice.

2. Add 8 μL of reverse transcription master mix (2 μL 10× first strand buffer, 4 μL dNTP mix, 1 μL RNase inhibitor, 1 μL Array script) to each tube and incubate the final 20 μL reaction at 42 °C for 2 h.
3. Briefly spin reactions and incubate on ice for 5 min.

3.6 Second Strand cDNA Synthesis

1. On ice, prepare second strand master mix (63 μL nuclease-free water, 10 μL 10× second strand buffer, 4 μL dNTP mix, 2 μL DNA polymerase, 1 μL RNase H) and add immediately to each sample.
2. Mix thoroughly by pipetting up and down and flicking the tube 3–4 times, and spin briefly to collect the reaction in the bottom of the tube. Incubate the final 100 μL reaction in a thermal cycler at 16 °C for 2 h.

3.7 cDNA Purification

1. Add 250 μL cDNA binding buffer to each sample and mix thoroughly as described above. After a short spin, proceed quickly to the next step.
2. Pass the mixture through a cDNA filter cartridge and centrifuge for 1 min at 10,000 × *g*. All the centrifugations in the purification procedure should be done at 10,000 × *g* at room temperature. Higher RCFs could cause mechanical damage or may deposit glass filter fiber in the eluate.
3. Apply 500 μL wash buffer to the cartridge and centrifuge for 1 min. Discard filtrate and centrifuge the filter cartridge for an additional minute to remove trace amounts of wash buffer.
4. Transfer the column to a new 2.0 mL tube and apply 18 μL nuclease-free water (preheated to 55 °C for at least 10 min) to the center of the filter in the cDNA cartridge.
5. Leave at room temperature for 5 min and centrifuge for 2 min at 10,000 × *g*. The double-stranded cDNA will now be in the eluate (~16 μL).

3.8 In Vitro Transcription to Synthesize aRNA

1. Add 24 μL IVT master mix (4 μL ATP, CTP, GTP, UTP (each 75 mM) and 4 μL of 10× reaction buffer and T7 enzyme mix) to each cDNA and mix thoroughly by pipetting and flicking the tube.
2. Incubate for 14–16 h (usually overnight) at 37 °C in a calibrated hybridization oven. We generally recommend long incubation times up to 16 h when working with small tissue amounts.
3. Add 60 μL nuclease-free water to each sample for adjusting the volume to 100 μL and to stop the reaction. Incubate the samples on ice and proceed to the aRNA purification step.

3.9 aRNA Purification

1. It is important for aRNA yield that the IVT reaction is exactly brought to 100 μL. Add 350 μL aRNA binding buffer and immediately 250 μL of 100% ethanol to each sample and mix by pipetting the mixture up and down three times. Do not vortex to mix and do not centrifuge! Pipet the mixture (700 μL) immediately to the center of the filter in the aRNA cartridge and centrifuge for 1 min. Any delay in this procedure could result in a loss of aRNA, because aRNA is in a semi-precipitated state when ethanol is added.
2. Wash with 650 μL wash buffer, centrifuge and discard the filtrate. Spin the column again for 1 min to remove traces of wash buffer and transfer the cartridge to a new 2.0 mL tube (provided RNA collection tube).
3. Add 100 μL of nuclease-free water to the center of the filter. Leave at room temperature for 5 min and centrifuge for 2 min. Purified aRNA will be eluted in 100 μL water.
4. RNA quantity and quality can now be assessed by using a fiber-optic spectrophotometer and microcapillary electrophoresis.

3.10 RNA-seq Output, Read Mapping, and Fidelity Confirmation of Expression Data

Amplified aRNA (100 ng) was used for TruSeq library preparation. As aRNA represents copies of mRNA, TruSeq RNA library preparation started with fragmentation of aRNA before proceeding with cDNA synthesis after the manufacturer's protocol (*see* **Note 5**). cDNA libraries were sequenced on Illumina HiSeq 2000. IVT-based libraries (ETC_1–3, Sync_1–3) generated 35–55 million paired-end reads (2× 100 bp) per sample (Table 1). High-sequence quality in all the samples is displayed by quality scores above 37. Between 84 and 89% of the reads could be mapped to the annotated barley reference genome [16]. From reads mapped to the genome, 60–70% hit barley high confidence (HC) genes that are physically anchored on the reference genome and represent coding regions/transcripts.

One main sticking point for interpreting data generated by multistep procedures is the reliability and reproducibility of the data. As it is well known that mRNA amplification introduces a certain bias in global transcriptome abundance, we evaluated the influence of mRNA amplification on RNA-seq data separately by comparison to non-amplified reference probes. Reference probes were generated from 250 to 300 microdissected sections of each tissue-type (ETC_0, Sync_0) and yielded around 400 ng total RNA, which was subsequently used in the standard TruSeq RNA sample preparation protocol. Comparison of reference with IVT-based samples showed for both tissues correlation coefficients ranging from 0.88 to 0.91 (Fig. 2c, d). High correlation between amplified and non-amplified samples confirmed minor effects of the amplification procedure on deduced expression values and thereby reflects the original mRNA profile to a large degree. High reproducibility of independent biological replicates is demonstrated by

Table 1
Quality of RNA-seq data from barley grain tissues isolated by LCM

Sample	Read output (paired-end)	Mean sequence quality[a]	Mapped reads (%)[b]	Hits HC genes (%)[c]
ETC_0/Ref	29.101.544	37.8	86.0	59.2
ETC_1/IVT	36.300.996	37.4	84.1	58.4
ETC_2/IVT	35.886.650	37.5	83.7	63.5
ETC_3/IVT	46.504.084	37.5	83.9	60.0
Sync_0/Ref	25.515.018	38.4	88.8	65.4
Sync_1/IVT	45.286.350	38.5	84.1	66.7
Sync_2/IVT	55.100.364	37.5	85.4	69.4
Sync_3/IVT	43.193.690	37.4	86.3	67.5

Read output, quality of the sequences, percentage of mapped reads to the barley genome and transcriptome is given for each sample

[a]Mean sequence quality given as Phred quality score which determines the base-calling accuracy. Values above 30 indicate a base call accuracy >99.9%

[b]Percentage of total reads mapped against Whole Genome Shotgun (WGS) sequencing assembly of barley cultivar 'Morex' (https://doi.org/10.1038/nature11543)

[c]High-confidence (HC) gene predictions derived from WGS sequences, based on barley transcripts and sequence homology to angiosperm proteins (26,159 genes)

representative scatter plots and correlation coefficients between 0.96 and 0.97 (Fig. 2b). Additionally, RNA-seq data were validated by qRT-PCR analysis. Transcript ratios of a selected subset of differentially expressed genes (DEGs) between ETC (1–3) and Sync (1–3)—some of them were identified as ETC-specific genes [12]—showed a high concordance between qRT-PCR and RNA-seq data (Fig. 3). In particular, obtained quantitative differences of transcript levels are overlapping with both methods to a high degree.

1. For data analysis, RNA-seq reads were mapped to the annotated barley reference genome with Tophat2 [17]. Read counts were quantified with HTseq [18] and normalized with DEseq2 [19] for the identification of DEGs.
2. Cluster dendrogram and heatmap were created from the Pearson correlation coefficients of normalized $\log_2$-transformed expression values using standard R functions (R Core Team 2015).
3. For qRT-PCR analysis, aRNA of the three biological replicates (ETC + Sync 1–3) was pooled in equimolar amounts for first strand cDNA synthesis using Superscript III (Invitrogen). Power SYBR Green PCR mastermix was used to perform reactions (5 technical replicates per gene) in an ABI 7900 HT Real-Time PCR system (Applied Biosystems). Log_2 fold-changes of

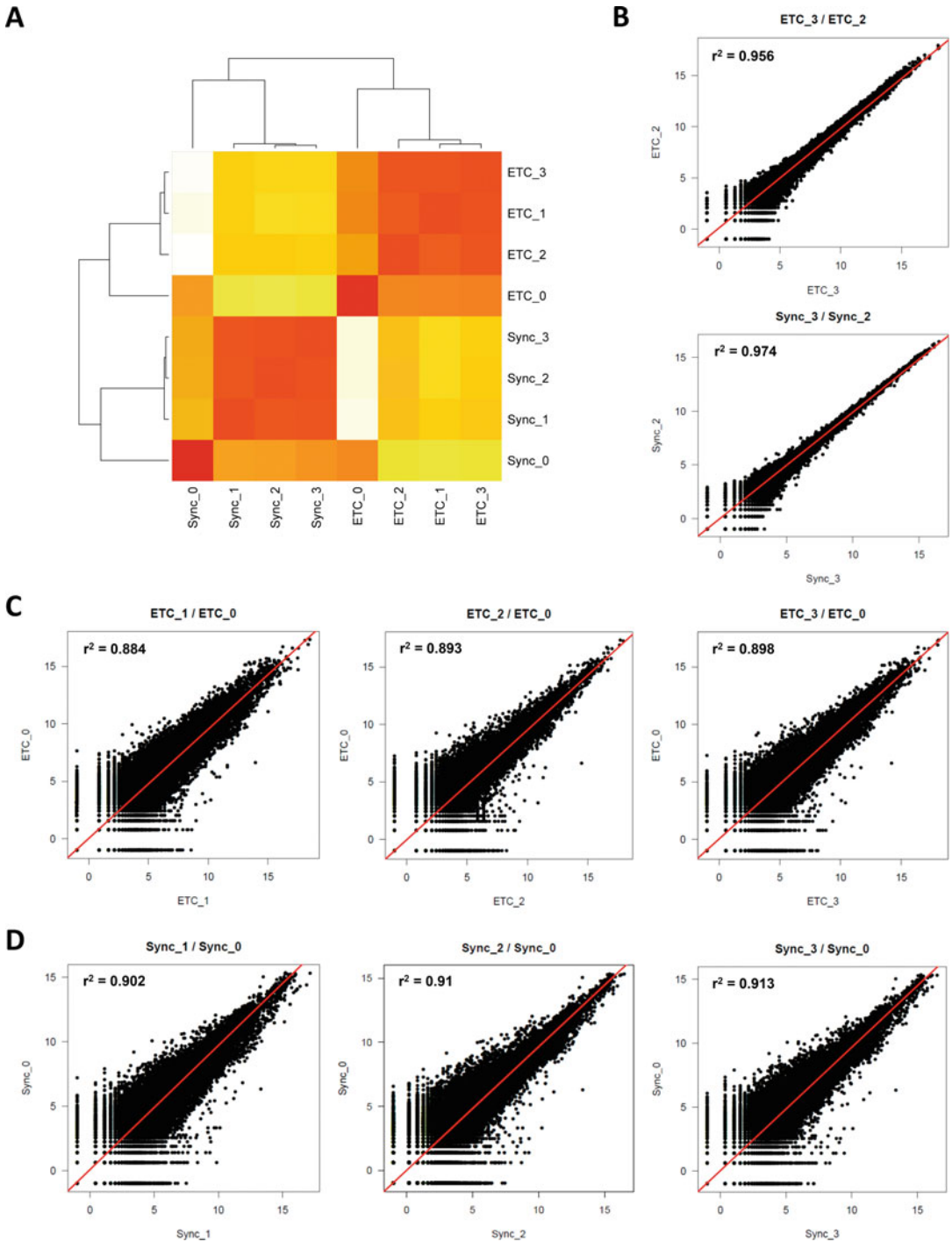

Fig. 2 Correlation and reproducibility of normalized $\log_2$-transformed expression values generated by RNA-seq of amplified and non-amplified/reference samples from microdissected grain tissues. Scatter plots display correlation between different samples with depicted correlation coefficients (r^2) in each plot. (**a**) Heat map display with configurated cluster dendogram shows Pearson correlation coefficients between the samples. The degree of color saturation corresponds to the level of correlation (light yellow—weak correlation, red—highest correlation).

qRT-PCR data were calculated after Livak and Schmittgen [20] as ΔΔCT-values using *HvActin1* (AK365182) as a reference gene.

In summary, analysis of RNA-seq data confirmed that the procedures described in this protocol are feasible for tissue-specific transcriptome analysis and generate highly reliable and reproducible data. Methods were also used for transcript analysis of different grain tissues and have been adopted for tissue-specific analysis of other plant organs, like spike meristems and floral organs of barley, supporting the general application and robustness of the protocol.

4 Notes

1. Despite cryosections do not meetthe same structural preservation as reached by tissue fixation and polymer embedding, we used cryosections because the morphological integrity from barley grains even at early developmental stages was sufficient for the identification and precise isolation of target tissues. Using frozen tissue without any further chemical treatment guarantees better quality and higher yield of extracted RNA compared to fixed material.
2. It is crucial for laser-assisted cutting and tissue collection that sections are totally dry. Any traces of moisture will inhibit transfer of microdissected cells and bear the risk of RNA degradation. If it is necessary to store cryosections or microdissected cells, use always sealed slide boxes in the presence of a desiccant (e.g., silica gel) for storage at −20 °C. In general, prolonged storage should be avoided and sample material should be used as fast as possible.
3. Complete lysis of sample material is mandatory for getting optimal yields and quality of extracted RNA. We recommend preheating the lysis buffer and incubating the microdissected cells in the lysis buffer for 15 min at 60 °C as our own experiments showed that the yield of total RNA is significantly

Fig. 2 (continued) Samples cluster generally into two main groups (ETC, Sync). Minor differences between non-amplified samples (ETC_0, Sync_0) and amplified samples (ETC + Sync_1–3) are depicted for a subgroup of genes by less color saturation. (**b**) Independent biological replicates of ETC and Sync tissues generated by IVT show a high correlation between 0.96 and 0.97. (**c**) Comparisons of IVT-amplified ETC samples (ETC_1–3) to a non-amplified reference sample (ETC_0) generated from 400 ng total RNA. (**d**) Comparisons of IVT-amplified Sync samples (Sync_1–3) to a non-amplified reference sample (Sync_0) generated from 400 ng total RNA. Correlation coefficients ranging from 0.88 to 0.91 between amplified and non-amplified samples indicate minor effects of the amplification procedure on deduced transcript abundancies

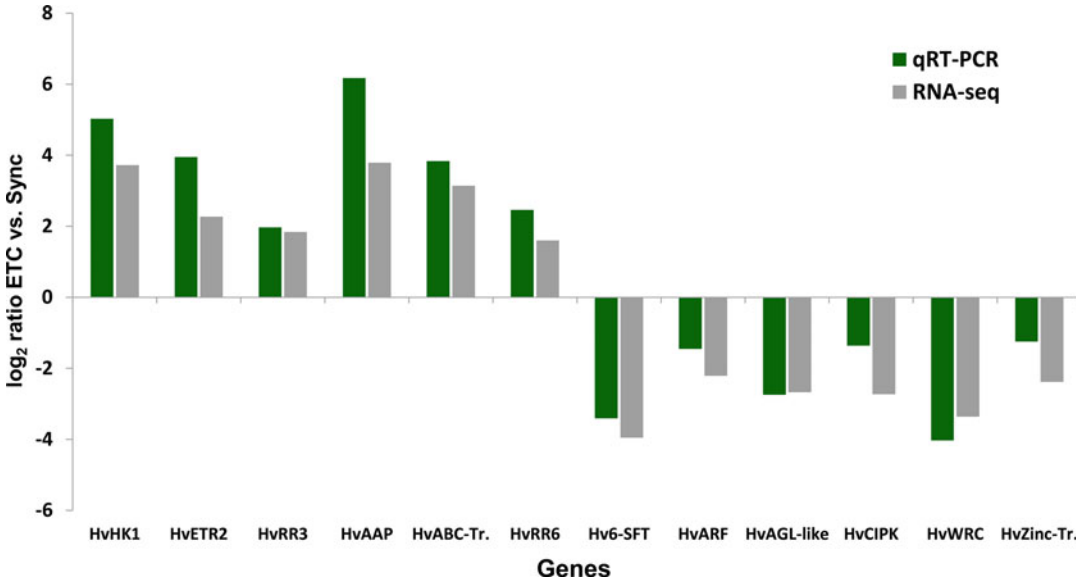

Fig. 3 Validation of RNA-seq data by qRT-PCR analysis. Expression of a subset of differentially expressed genes (DEGs) between ETC and Sync extracted by RNA-seq analysis was assessed by qRT-PCR. Transcript ratios showed a high concordance between qRT-PCR and RNA-seq data confirming the reliability of the procedure for RNA-seq analysis based on LCM-isolation of barley grain tissues

increased upon heat incubation. The lysis buffer contains guanidine thiocyanate which prevents RNase activity.

4. A precise estimation of RNA yields from these minimum tissue amounts is rather difficult. Based on our experimental data from fiberoptic spectrophotometer and microcapillary electrophoretic analyses, the yield of total RNA from microdissected tissues generally ranges between pictogram amounts to 5 ng RNA. More reliable results about RNA quantities can be achieved after the first round of amplification.
5. TruSeq library preparation starts with 100 ng of aRNA. Because aRNA represents copies of mRNA, the used amount corresponds to mRNA concentrations obtained from 3.3 to 10 μg of total RNA (1–3% of total RNA). Concomitantly, the mRNA purification step in the TruSeq v2 protocol was skipped and library preparation started with fragmentation of aRNA in a maximal volume of 5 μL before proceeding after the manufacturer's protocol.

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